

Endogenous GDNF as a Regulator of Midbrain Dopamine Neurons

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”Tee äärimmäinen kysymys,
tee se joka päivä.”

A.W. Yrjänä: Pyydä Mahdotonta (1990)

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ABSTRACT

Midbrain dopamine neurons exert a powerful influence on behavior and their dysfunction is associated with many neurological and neuropsychiatric diseases, including Parkinson's disease (PD). Dopamine neurons are large, complex and sensitive cells. Hence, their survival and correct function requires coordinated action of various transcription and regulatory factors both during development and aging. Potentially, one such factor is glial cell line-derived neurotrophic factor (GDNF). Ectopically applied GDNF is best known for its potent ability to protect and restore damaged dopaminergic neurons both *in vitro* and *in vivo*. GDNF-based therapies have been tested in clinical trials with PD patients with variable success. However, the function of endogenous GDNF in brain dopamine system development, aging and disease is poorly understood. Improvement in GDNF-based therapies requires better understanding of the physiological functions of GDNF in the brain.

The current knowledge of endogenous GDNF function remains obscure, mainly due to the lack of proper animal models. The present study investigated the regulatory role of endogenous GDNF in the development, maintenance and function of midbrain dopamine neurons utilizing novel mouse models: GDNF conditional knock-out (cKO) mice and GDNF hypermorphic (GDNFh) mice over-expressing GDNF from the endogenous locus. GDNF cKO mice enable GDNF deletion solely from the central nervous system during embryonic development or later in adulthood, preserving its vital role in kidney development. Midbrain dopamine systems of these new mouse strains were studied with immunohistochemical, neurochemical, pharmacological, behavioral and molecular biology methods.

We found more substantia nigra dopaminergic cells and elevated striatal dopamine levels in immature and adult GDNFh mice. In cKO mice, dopamine levels and cell numbers were unaltered, even upon aging, and regardless of the timing of GDNF deletion. Both mouse strains exhibited enhanced dopamine uptake, while responses to amphetamine were augmented in GDNFh mice and reduced in cKO mice. GDNFh mice also released more dopamine and GDNF elevation protected them in a lactacystin-based model of PD. Overall, dopamine neurons were more sensitive to moderate elevation than complete absence of endogenous GDNF, which suggests that they can adaptively compensate for GDNF loss. This highlights the limitation of broadly utilized gene deletion approaches in analyzing gene function.

Our results indicate a clear role for endogenous GDNF in midbrain dopamine neuron development and function, but also demonstrate that GDNF is not required for their maintenance during aging. Furthermore, the ability of endogenous GDNF to protect animals in a PD model without the side effects associated with ectopic GDNF application suggests that elevation in endogenous GDNF levels may be an important future route for PD therapy.

TIIVISTELMÄ

Aivojen dopamiinihermosoluilla on voimakas vaikutus käyttäytymiseemme ja niiden toimintahäiriö onkin liitetty moniin neurologisiin ja psykiatrisiin sairauksiin, kuten Parkinsonin tautiin. Dopamiini neuronit ovat suuria, monimutkaisia ja herkkiä soluja. Tämän vuoksi niiden selviytyminen ja oikeanlainen toiminta niin yksilönkehityksen kuin koko elinkaaren ajan on riippuvaista useiden erilaisten säätelytekijöiden oikeanlaisesta yhteistoiminnasta. Mahdollisesti eräs tällainen säätelytekijä on gliasolulinjaperäinen hermokasvutekijä eli GDNF. GDNF:llä on osoitettu olevan hyvin poikkeuksellinen kyky suojella ja korjata vaurioituneita dopamiinihermosoluja sekä solu- että eläinmalleissa. GDNF-peräisiä lääkkeitä onkin tutkittu kliinisissä kokeissa Parkinsonintautipotilailla, vaihtelevin tuloksin. Tästä huolimatta endogeenisen, eli aivojemme itse valmistaman, GDNF:n toiminta yksilönkehityksen, vanhenemisen ja sairauksien yhteydessä tunnetaan yhä huonosti. Tehokkaampien GDNF-pohjaisten hoitojen kehittäminen edellyttää parempaa ymmärrystä GDNF:n fysiologisista toiminnoista aivoissa.

Endogeenisen GDNF:n toimintojen heikko tuntemus johtuu ensisijaisesti kunnollisten eläinmallien puuttumisesta. Tässä työssä tutkimme endogeenisen GDNF:n roolia keskiaivojen dopamiinihermosolujen kehityksessä, ylläpidossa ja toiminnassa käyttäen uusia eläinmalleja: konditionaalisesti poistogeenisiä (conditional knock-out; cKO) GDNF hiiriä sekä GDNF hypermorfisia (GDNFh) hiiriä, jotka tuottavat normaalia enemmän endogeenistä GDNF:ää. cKO hiiriltä GDNF voidaan sikiövaiheessa poistaa täysin ainoastaan keskushermostosta tai vaihtoehtoisesti vasta myöhemmin aikuisilta eläimiltä. Näin säilytetään GDNF:n elintärkeä rooli munuaisten kehityksessä. Tutkimme näiden uusien hiirikantojen keskiaivojen dopamiinijärjestelmiä immunohistokemiallisten, aivokemiallisten, farmakologisten, molekyylibiologisten sekä erilaisten käyttäytymismenetelmien avulla.

Havaitsimme sekä hyvin nuorten että aikuisten GDNFh hiirten aivoissa kohonneen määrän dopamiinia sekä dopamiinihermosoluja. Toisaalta GDNF cKO hiirillä dopamiinipitoisuudet ja -solumäärät säilyivät muuttumattomia, jopa hyvin vanhoilla hiirillä, ja riippumatta GDNF:n poistamisen ajankohdasta. Molemmilla hiirikannoilla dopamiinin takaisinotto oli voimistunut, kun taas amfetamiinivasteet olivat vahvistuneet GDNFh hiirillä ja heikentyneet GDNF cKO hiirillä. GDNFh hiirillä dopamiinia myös vapautui enemmän, minkä lisäksi kohonneet GDNF-pitoisuudet suojasivat niitä kemiallisesti aiheutetulta Parkinsonismilta. Kaiken kaikkiaan aivojen dopamiinihermosolut näyttivät olevan herkempiä GDNF:n määrän kohtuulliselle lisääntymiselle kuin sen täydelliselle puuttumiselle. Dopamiinihermosolut kykenevät siis ilmeisesti jollain tavalla kompensoimaan GDNF:n puuttumisen. Tämä osoittaa selvän puutteen hyvin yleisesti käytetyissä geeninpoistomenetelmissä.

Tuloksemme viittaavat siihen että endogeenisellä GDNF:llä on selvä rooli aivojen dopamiinihermosolujen kehityksessä ja toiminnassa. Toisaalta tuloksemme myös osoittavat, ettei GDNF:ää välttämättä tarvita ylläpitämään niitä yksilön vanhetessa. Lisäksi endogeenisen GDNF:n kyky suojella eläimiä Parkinsonin tautimallissa ilman GDNF-annosteluun tavallisesti liittyviä sivuvaikutuksia merkitsee, että endogeenisen GDNF:n lisääminen saattaisi joskus tulevaisuudessa olla tehokas tapa hoitaa Parkinsonin tautia.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (I-III):

- I Kopra J*, Vilenius C*, Grealish S, Härma M-A, Varendi K, Lindholm J, Castrén E, Vöikar V, Björklund A, Piepponen TP, Saarma M, Andressoo J-O (2015). GDNF is not required for catecholaminergic neuron survival in vivo. *Nat Neurosci* 18(3):319-322.
- II Kopra J*, Panhelainen A*, af Bjerken S, Porokuokka L, Montonen H, Piepponen TP, Saarma M, Andressoo J-O. Altered dopamine transporter function and amphetamine-stimulated behavior in the absence of brain GDNF. (submitted)
- III Kumar A*, Kopra J*, Varendi K*, Porokuokka L, Panhelainen A, Kuure S, Marshall P, Nevalainen N, Härma M-A, Vilenius C, Lilleväli K, Tekko T, Mijatovic J, Pulkkinen N, Jakobson M, Jakobson M, Ola R, Palm E, Lindahl M, Strömberg I, Vöikar V, Piepponen TP, Saarma M, Andressoo J-O (2015). GDNF overexpression from the native locus reveals its role in the nigrostriatal dopaminergic system function. *PLoS Genet* 11(12): e1005710.

* Equal contribution

The publications are referred to in the text by their roman numerals. Reprints were made with the permission of the copyright holders.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
ANOVA	Analysis of variance
ARTN	Artemin
BDNF	Brain-derived neurotrophic factor
cKO	Conditional knock-out
CNS	Central nervous system
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
dSTR	Dorsal striatum
GABA	γ -aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GDNFh	GDNF hypermorphic
GFL	GDNF family ligand
GFR α	GDNF family receptor alpha
GPe	Globus pallidus external segment
GPI	Globus pallidus internal segment
GPI	Glycosyl phosphatidylinositol
HVA	Homovanillic acid
i.p.	Intraperitoneally
MEN2B	Multiple endocrine neoplasia type 2 B
MPTP	1-Methyl-4-phenyl-1,2,3,6- tetrahydropyridine
mRNA	messenger ribonucleic acid
NCAM	Neural cell adhesion molecule
NO	Nitric oxide
NRTN	Neurturin
PD	Parkinson's disease
PSPN	Persephin
PV	Parvalbumin
Ret	Rearranged during transfection
SEM	Standard error of mean
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TGF- β	Transforming growth factor- β
TH	Tyrosine hydroxylase
VMAT2	Vesicular monoamine transporter 2
vSTR	Ventral striatum
VTA	Ventral tegmental area

1 INTRODUCTION

In the absence of external signals most cells quickly die through an active process of programmed cell death. In multicellular organisms this internal ‘death signal’ is normally counterbalanced by external survival and growth signals. These signals not only keep the right cells alive but also regulate the size, shape, formation and function of different tissues and organs. Our body synthesizes a large number of different growth factors – small, secreted proteins – that deliver survival and growth signals for the cells that carry cognate receptors on their surface. The brain is no exception to this generality.

Neurotrophic factors comprise a superfamily of growth factors that regulate the life of neurons, being involved in almost every aspect of their lifecycle from development to death. Glial cell line-Derived Neurotrophic Factor (GDNF) is best known for its ability to support midbrain dopaminergic neurons (Lin et al., 1993), although it also regulates certain other neuronal populations and has critically important functions outside the nervous system, such as regulation of kidney and enteric nervous system development (Airaksinen and Saarma, 2002). Midbrain dopamine neurons regulate some very important aspects of our behavior including motivation, attention, associative learning, emotions, cognition and initiation of movements.

Parkinson’s disease is an example of a serious disease, where inadequate dopamine system function plays an important role. Due to its potent dopaminotrophic effects, GDNF has been extensively studied as a potential novel disease-modifying drug candidate for the dopaminergic neurodegeneration, which causes many of the classical motor symptoms of Parkinson’s disease. These studies have taken intracranial ectopic GDNF application all the way into clinical trials with Parkinson’s disease patients with variable success and new clinical studies again ongoing (Olanow et al., 2015). While most attention has been directed to the ability of ectopic GDNF to recover and save damaged dopamine neurons, its effects on intact dopamine neurons has received less attention. Furthermore, the focus on the potential therapeutic aspects of ectopically applied GDNF protein has often blurred the fact that the exact role and functions of endogenous, physiological GDNF, especially in the adult brain, are still rather unknown. The main reason for this is that the complete removal of GDNF from the body via GDNF gene deletion causes death very soon after birth due to kidney agenesis and lack of enteric nervous system distal from the stomach (Pichel et al., 1996; Airaksinen and Saarma, 2002).

In the present study, we have used novel and innovative *in vivo* approaches to circumvent this problem in order to uncover the functions of endogenous GDNF in the brain and elsewhere in the body. This thesis provides new knowledge about the role of endogenous GDNF as a regulator of midbrain dopamine neurons.

2 REVIEW OF THE LITERATURE

2.1 The brain dopamine systems

Based on their pioneering work in the late 1950s, Arvid Carlsson and coworkers proposed that dopamine would also act as an independent neurotransmitter in the brain, instead of being a mere precursor for noradrenaline and adrenaline (Carlsson et al., 1957, 1958; Carlsson, 1959). Since that time, brain dopamine pathways have been subjects of intensive studies. Later discoveries that dopamine pathways regulate many important behaviors and associate to the pathogenesis of various diseases further fueled the scientific interest towards brain dopamine systems.

Dopamine pathways regulate motivated behavior through their role in reward signaling and thus have a central role in behavioral reinforcement and associative learning. Striatal dopamine signaling creates associations between a context and a meaningful outcome. This context can be either external, like a particular environmental cue, or internal, like a particular behavior or a set of behaviors. The resulting outcome can be either positive leading to reinforcement or negative leading to aversion. Repeated reinforcements of behavior lead to generation of an automatic motor program (a theoretical representation of planned movements) or a habit. During this process, the neuronal control of behavior shifts from ventral to dorsal striatum. If this habit formation is particularly strong due to repeated, strongly reinforcing stimuli the resulting behaviors may become compulsive, as happens in most addictions. These neural mechanisms are reviewed more comprehensively by (Wise, 2004).

In addition to learning and acquisition of motor programs, dopamine plays an important role in the selection and initiation of appropriate behavioral responses (decision-making), regulation of attention, emotions and working memory as well as moment-to-moment motor control and motor programming.

The significance of brain dopamine systems is highlighted by the fact that the deterioration of their normal function is associated with various neurological and psychiatric disorders like Parkinson's disease (PD), Huntington's disease, depression, schizophrenia, ADHD, bipolar disorder, compulsive disorder as well as various addictions.

2.1.1 *Midbrain dopamine neurons and their connectivity*

In the mammalian mesencephalon dopamine neuron cell bodies are located in substantia nigra pars compacta (SNpc), ventral tegmental area (VTA) and the retrorubral field (RRF), which respectively correspond to the cell groups A9, A10 and A8 (Dahlström and Fuxe, 1964; Fuxe, 1965). These neurons send their long axons via the medial forebrain bundle to the cortical, limbic and striatal areas of the brain (Figure 2.1). Whereas in rodent neocortex dopaminergic innervation is limited to the frontal, cingulate and entorhinal cortex, in primates (like humans) the entire cortical mantle receives dopaminergic innervation (Berger et al., 1988, 1991; Gaspar et al., 1989; Meador-Woodruff et al., 1996). The limbic areas innervated by the dopamine neurons in A10 are ventral striatum (including nucleus accumbens), amygdala, olfactory tubercle and septum. The dorsal sensorimotor compartment of striatum (or caudate-putamen) receives dopaminergic innervation almost exclusively from the SNpc A9 group of neurons that form the nigrostriatal pathway (Andén et al., 1964; Dahlström and Fuxe, 1964; Dahlström et al., 1964; Hokfelt and Ungerstedt, 1969; Ungerstedt, 1971). Similarly, the connections from RRF A8 and VTA A10 dopamine neurons to the limbic and cortical structures form the mesocorticolimbic

pathway that is often separated into mesolimbic and mesocortical tracts (Dahlström et al., 1964; Ungerstedt, 1971; Thierry et al., 1973; Swanson, 1982; Björklund and Dunnett, 2007). There are several molecular markers that are differently expressed by the A9 and A10 cell groups: A9 neurons express aldehyde dehydrogenase 2 (Ald2) and G protein-regulated inward rectifier K⁺ channel subfamily-J member-6 (Girk2/KCNJ6), while A10 neurons predominantly express the calcium-binding proteins calbindin 1 and 2 (Calb1 and 2) and cholecystokinin (CCK) (Veenvliet et al., 2013). Further anatomical and functional diversity of midbrain dopamine systems is beginning to be defined as novel research tools allow characterization of new dopaminergic subpopulations within the SNpc and VTA (Reviewed by Roeper 2013). The mouse midbrain contains around 20,000-30,000 dopamine neurons, out of which ~50 % reside in the SNpc (Björklund and Dunnett, 2007). This thesis focuses mainly on SNpc dopamine neurons that form the nigrostriatal dopaminergic system.

A recent study revealed that SNpc dopamine neurons have extremely wide and dense axonal arborizations enabling a single neuron to cover up to 6 % of the total volume of rat striatum (Matsuda et al., 2009). Consequently, it was estimated that around 75,000 striatal neurons are directly influenced by a single dopamine neuron. While a single neostriatal neuron is estimated to be simultaneously under the influence of 95-194 dopaminergic neurons (Matsuda et al., 2009), this raises an obvious question of the implications for this exceptional degree of overlap and redundancy in the dopaminergic innervation. The authors speculated that the answer might be in an inherent lability of dopaminergic neurons that needs to be compensated by high safety margins. This overlap might also be necessary for learning or fine-tuning complex motor programs.

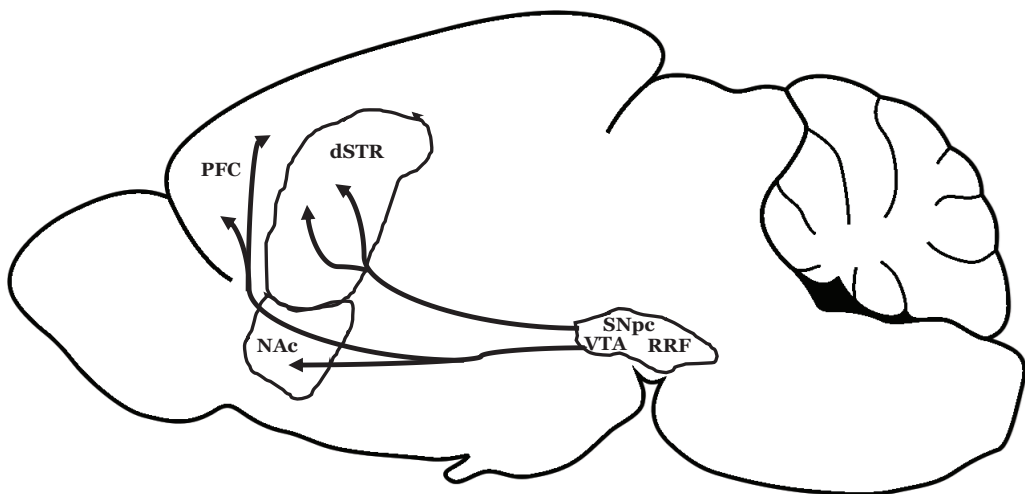


Figure 2.1. Dopaminergic forebrain projections from the ventral midbrain. Schematic presentation of the main dopaminergic projections from substantia nigra pars compacta (SNpc, A9), ventral tegmental area (VTA, A10) and the retrorubral field (RRF, A8). These neuronal groups send major axonal projections to dorsal striatum (dSTR) as well as nucleus accumbens (NAc) and prefrontal cortex (PFC). These long dopaminergic projections are called nigrostriatal and mesocorticolimbic tracks, respectively.

Dopaminergic neurons have an intrinsic pacemaker activity that allows them to continuously release low amounts of dopamine (Grace and Bunney, 1984). Synaptic inputs then modify this tonic firing pattern in response to internal and external stimuli causing either transient pauses or phasic bursts in activity (Bunney et al., 1973a, 1973b; Grace and Bunney, 1984; Lee and Tepper, 2009; Tritsch and Sabatini, 2012). An elegant and comprehensive study mapped all the direct, monosynaptic inputs to the SNpc and VTA dopamine neurons in mouse brain (Watabe-Uchida et al., 2012). The study largely confirmed and further refined the known rich connectivity from the basal ganglia and the significant connectivity from many other brain structures to the dopamine neurons. Perhaps most interestingly, the monosynaptic inputs solely to the SNpc neurons came from somatosensory and motor cortices as well as from subthalamic nucleus (STN) and dorsal striatum, while the VTA neurons received exclusive inputs from hypothalamus, lateral orbital cortex and ventral striatum (Watabe-Uchida et al., 2012).

Importantly, dopamine is not only released from the axonal terminals, but also from the somatodendritic areas of the SNpc (and VTA) and from the dendrites that extend throughout large parts of substantia nigra pars reticulata (SNpr) (Björklund and Lindvall, 1975; Robertson and Robertson, 1989; Robertson et al., 1991; Cragg et al., 1997; Rice et al., 1997; Hoffman and Gerhardt, 1999). As SNpr functions as a basal ganglia output nucleus (reviewed later below) the direct dopaminergic regulation of motor behaviors also takes place via somatodendritic release (Rice et al., 2015). In addition, the dopaminergic dendrites extending to SNpr receive more GABAergic inhibitory innervation than the dendrites in the SNpc region which contributes to the specific firing patterns of different SNpc neurons (Henny et al., 2012). Somatodendritic dopamine also regulates the firing activity in neighboring dopamine neurons via inhibitory dopamine D2 autoreceptors (Rice et al., 2015). This mechanism shapes the patterns of release in the axonal terminals (Rice et al., 2015).

2.1.2 Midbrain dopamine neuron development

The full complement of midbrain dopamine neurons takes place in multiple phases that include specification of the neuronal field where the dopamine cells will form, cell differentiation and migration of the immature dopamine neuron precursors to their specific positions, axonal outgrowth and connectivity, selective programmed cell death, and finally maintenance of the mature dopamine neurons (Smidt and Burbach, 2007). Further importance of understanding dopamine neuron development comes from the fact that many of the developmental factors are also important for the function, plasticity and maintenance of these neurons during adulthood and aging.

Dopamine neuron neurogenesis takes place approximately between embryonic day (E) 9.5 and E14.5 in the mouse embryo (Luo and Huang, 2015). The main external determinants in the initial patterning process are fibroblast growth factor 8 (FGF8) produced by the isthmus and the morphogen sonic hedgehog (Shh) initially secreted by the notochord. Intersection of these two signals defines the neuronal field within the ventricular zone where the midbrain dopamine neurons are born, but the process also requires transforming growth factor β (TGF β) and WNT (mainly Wnt1 and Wnt5a) signaling (Smidt and Burbach, 2007; Arenas et al., 2015; Luo and Huang, 2015). After the initial patterning, the expression of several cell type-specific transcription factors that include Nurr1, Pitx3, Engrailed-1/2 (En1/2), Otx2, Foxa1/2, Ng2, Mash1, Msx1, LXR α/β and Lmx1a/b are needed for the progenitor cells to finally attain the proper dopaminergic identity (Smidt and Burbach, 2007; Alavian et al., 2008; Arenas et al., 2015).

Cell migration from the ventricular zone to the final destination at the marginal zone can be seen as part of the differentiation program and they indeed occur simultaneously (Arenas et al., 2015). This migration is regulated by several factors, most important being C-X-C motif chemokine 12 (CXCL12) and its receptor C-X-C motif chemokine receptor type 4 (CXCR4) that control radial migration and RELN (reelin) signaling that controls tangential migration (Arenas et al., 2015).

Out of the transcription factors mentioned above the 'late' transcription factors Nurr1, Pitx3 and En1/2 control the acquisition of mature dopaminergic phenotype and remain expressed until adulthood. They regulate each other's expression as well as the expression of many genes that define the mature dopaminergic neuron including TH, VMAT2, DAT, AADC, D2 receptor and GDNF receptor Ret (Arenas et al., 2015). Interestingly, Nurr1 seems to be required for the proper development of all midbrain dopamine neurons, while Pitx3 seems to be more important for SNpc cells and En1 for VTA neurons (Veenvliet et al., 2013). It appears that the crosstalk between these two factors strongly influences the specification of the two midbrain dopamine neuron subsets, SNpc and VTA neurons, during development (Veenvliet et al., 2013). Other key regulators of this differentiation process appear to be Otx2-Wnt1-Lmx1a/b and Shh-Foxa1/2 pathways, specifying VTA and SNpc neurons respectively (Arenas et al., 2015).

Once established, the different midbrain dopamine neuron systems need to be maintained throughout the entire adult life. This is achieved by differential expression of transcription factors and other key regulators, like neurotrophic factors, which will be summarized in a later section. For example, in addition to their crucial roles in terminal differentiation, Nurr1, Pitx3, En1/2 and Otx2 also regulate dopamine neuron maintenance and survival until the end of embryonic development and throughout the postnatal life (Alavian et al., 2008; Di Salvio et al., 2010; Arenas et al., 2015).

Striatum and cortical areas receive dopaminergic innervation by mid-to-late gestation, significantly earlier in primates than in rodents (Money and Stanwood, 2013). In rats this begins at E14 (Specht et al., 1981) and in humans as early as 6-7th gestational week (Verney et al., 1991; Zecevic and Verney, 1995). This coincides with striatal and cortical neurogenesis and differentiation and dopamine critically modulates the developmental processes in these regions (Money and Stanwood, 2013). During development, most neuronal populations go through a natural, programmed and regulated cell death process that can reduce their initial numbers by over half (Cowan et al., 1984). As an apparent part of the normal maturation process apoptotic cell death also takes place in the SNpc dopamine neurons of rats (Janec and Burke, 1993; Oo and Burke, 1997) and mice (Jackson-Lewis et al., 2000). In mice the developmental apoptosis of dopamine neurons peaks at postnatal days (P) 2 and P14 and is largely over by P30 (Jackson-Lewis et al., 2000). The process is dependent on the target structure, the striatum, and most likely regulates adequate and effective target innervation (Jackson-Lewis et al., 2000). Hence, dopamine neuron development and maturation continue postnatally. This period (especially adolescence) is suggested to be associated with particular behaviors (impulsivity, sensation seeking), vulnerabilities and onset of many neuropsychiatric diseases (Money and Stanwood, 2013).

2.1.3 Dopamine lifecycle

Dopamine challenges the classical view of neurotransmitters as short-lived molecules that relay quick and precise point-to-point signals over synaptic clefts and are then quickly degraded

or taken up by the neighboring cells. Instead the dopamine signal is a relatively slow (active lifetime 10-100 ms) and diffuse signal that spreads extensively in the extracellular space reaching receptors far away from the release site (up to 7 μm) (Ungerstedt et al., 1969; Agnati et al., 1995; Rice and Cragg, 2008; Fuxe et al., 2015). Dopamine signal is not designed to be precise. In fact most axonal dopamine release terminals completely lack specialized post synaptic structures (Wilson et al., 1977; Descarries et al., 1996; Rice et al., 2011; Taber and Hurley, 2014). A recent electron microscopy study estimated synaptic incident of dopamine terminals to be around 5 % in the mouse striatum (Bérubé-Carrière et al., 2012). It is estimated that after a quantal release dopamine can encounter ~300-2500 synapses (depending on the quantal size) in the striatum until its concentration falls too low to activate high affinity receptors (Rice and Cragg, 2008). This sort of extracellular fluid-mediated ‘volume transmission’ is typical for all the monoamine transmitters and often changes the activity state of larger brain areas and can result in changes in mood, attention or alertness (Taber and Hurley, 2014; Fuxe et al., 2015). Interestingly, there is convincing new evidence that dopamine neurons can co-release γ -aminobutyric acid (GABA) (Tritsch et al., 2012, 2014, 2016; Stamatakis et al., 2013) and glutamate (Yamaguchi et al., 2011; Broussard, 2012; Li et al., 2013; Zhang et al., 2015), but the exact significance of this phenomenon is unclear.

Although dopamine neuron firing is the main determinant of striatal dopamine release, it alone fails to provide sufficient local specificity to the dopamine signal, as a single dopamine neuron covers up to 6 % of total striatal volume with its axonal tree (Matsuda et al., 2009). The high level of temporal and spatial regulation observed in the striatum results from diverse local regulatory and gating mechanisms for dopamine release. Thus, striatal dopamine release is driven and regulated at two independent levels: distantly, through SNpc/VTA firing activity, and locally at the striatal level through reuptake, autoreceptor- and heteroreceptor-dependent modulation, as well as termino-terminal and local network (striatal interneurons) control (Rice et al., 2011; Cachope and Cheer, 2014; Sulzer et al., 2016)A.

In most brain areas the dopamine signal is not terminated by degradation or rapid uptake, but primarily by passive diffusion and only secondarily by active reuptake back into the dopaminergic neurons by a specific dopamine transporter (DAT) (Cragg and Rice, 2004; Rice and Cragg, 2008). This is evidenced by small effects of uptake inhibition on the “effective radius” (7 μm vs. 8.2 μm), where dopamine concentration after quantal release remains high enough to activate high affinity dopamine (D2) receptors (Cragg and Rice, 2004; Rice and Cragg, 2008). However, the small difference in effective radius still means that the released dopamine will activate 40 % lower number of D2 receptors within its sphere of influence (Rice and Cragg, 2008). Uptake has an even smaller impact on the effective radius or the sphere of influence of the low affinity dopamine (D1) receptors (Rice and Cragg, 2008). Importantly, DAT has a much greater influence on larger dopamine transients that result from release of multiple vesicles due to burst firing or summation of multiple release sites (Floresco et al., 2003; Rice and Cragg, 2008). Finally, the main role of striatal DAT is to limit dopamine lifetime after release, which directly influences dopamine transmission (Cragg and Rice, 2004; Rice and Cragg, 2008). In mice lacking DAT, dopamine persists 100 times longer in the extracellular space and various compensatory changes ensue (Giros et al., 1996; Jones et al., 1998; Jaber et al., 1999). Thus, the key role of DAT is not in the termination of normal dopamine signal, but in dopamine clearance and recycling that maintain homeostasis and in the control of larger dopamine transients. In comparison to the glutamate transporter that exists primarily on non-glutamatergic cells, like astroglial cells

(Seal and Amara, 1999; Danbolt, 2001), DAT is exclusively expressed on the surface of dopamine neurons (Ciliax et al., 1995; Nirenberg et al., 1996, 1997; Hersch et al., 1997) and its uptake rate is around ten times slower (Wadiche et al., 1995; Povlock and Schenk, 1997; Prasad and Amara, 2001). DAT expression is also primarily extrasynaptic and the dopamine uptake sites are rather evenly distributed along the surface of dopaminergic fibers (Nirenberg et al., 1996; Pickel et al., 1996; Hersch et al., 1997). Due to its slow kinetics and limited expression DAT cannot compete with the quick escape of dopamine from the site of release by diffusion (Cragg and Rice, 2004; Rice and Cragg, 2008). This allows dopamine to reach receptors far away from the site of release. Several types of proteins including kinases, receptors and scaffolding proteins interact with DAT, modulating either its catalytic activity or cellular membrane trafficking (Eriksen et al., 2010).

An important level of control for dopamine signaling comes from its highly regulated biosynthetic pathway, where amino acid tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) and further to dopamine. Cytosolic enzyme tyrosine hydroxylase (TH) catalyzes the first (Nagatsu et al., 1964) and rate-limiting (Levitt et al., 1965) step of this pathway, while aromatic L-amino acid decarboxylase (AADC) catalyzes the second. As an indication of its importance, the amount and enzymatic activity of TH are tightly regulated at transcriptional, translational and post translational levels (Tekin et al., 2014). The most dynamic regulation on TH activity comes from the direct feedback inhibition by dopamine as well as from phosphorylation and dephosphorylation of its three serine residues (Ser19, 31 and 40) (Tekin et al., 2014), which regulate synthesis.

As all catecholamines (dopamine, noradrenaline and adrenaline) react with oxygen at neutral pH to generate toxic derivatives, they are mostly synthesized on demand and stored in acidic synaptic vesicles (Graham et al., 1978; Hastings and Zigmond, 1994; Hastings et al., 1996; Tekin et al., 2014). Therefore any uptaken or newly synthesized dopamine is subsequently packed into storage vesicles by the vesicular monoamine transporter 2 (VMAT2) or alternatively metabolized by enzymes monoamine oxidase (MAO) and aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid (DOPAC) (Eisenhofer et al., 2004). DOPAC is then further metabolized by catechol-O-methyl-transferase (COMT) into homovanilic acid (HVA), which is ultimately secreted in urine (Elchisak et al., 1982). Contrary to the common view, most of the dopamine turnover and metabolism takes place within the dopaminergic cells due to the constitutive passive leakage from the storage vesicles to the surrounding cytoplasm independently of exocytotic release (Floor et al., 1995; Eisenhofer et al., 2004). This leakage is mostly balanced by active, energy-consuming transport back into the vesicles and only a small fraction (~10 %) of dopamine escapes vesicular sequestration by VMAT2. However, this fraction represents a major source of dopamine metabolites (Eisenhofer et al., 2004). VMAT2 activity therefore strongly affects dopamine storage, as is also indicated by VMAT2 heterozygous mice with increased tissue dopamine levels (Takahashi et al., 1997). VMAT2 also protects neurons from the cytosolic toxicity of dopamine and other catecholamines (Eiden and Weihe, 2011). High dopamine content in the nigrostriatal dopamine system may predispose these neurons to disturbances as it results in particularly high rates of vesicular leakage and energy consuming sequestration back into the vesicles (Eisenhofer et al., 2004).

2.1.4 Dopamine receptors and modulatory effects

The dopamine signal is received by dopamine receptors, which belong to the family of metabotropic G-protein coupled receptors (GPCRs). Dopamine receptors are primarily, but not exclusively, located outside synapses and often some distance away from the dopamine release sites (Sesack et al., 1994; Hersch et al., 1995; Yung et al., 1995; Khan et al., 1998). The five dopamine receptors are divided into two subclasses based on their structural, pharmacological and signaling properties: D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4). D1 and D2 receptors are the most abundant subtypes in the brain, D1 displaying the widest distribution and highest expression (Jaber et al., 1997). The expression of the other subtypes (D3, D4 and D5) is substantially more restricted and less dense (Jaber et al., 1997). The affinity of D2-like receptors for dopamine is 10 to 100 fold higher than that of D1-like receptors with values of ~10 nM for D2 versus ~1 μ M for D1 (Richfield et al., 1989; Beaulieu and Gainetdinov, 2011; Sulzer et al., 2016). Hence, the basal extracellular levels of dopamine (10-20 nM) would only activate D2 receptors. D1 receptors are positively coupled to adenylyl cyclase (Brown and Makman, 1972; Kebebian et al., 1972; Kebebian and Calne, 1979), leading to production of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA), which phosphorylates various intracellular targets. By contrast, D2-like receptors are negatively coupled to adenylyl cyclase (Giannattasio et al., 1981; Onali et al., 1983; McDonald et al., 1984; Enjalbert et al., 1986, 1990), and activate protein phosphatases that directly counter the effects of PKA. Thus, these two signaling pathways have primarily opposite cellular effects.

The effects and mechanisms of dopamine signaling are highly complex and not completely understood. Dopamine downstream signaling includes a variety of molecules such as phosphatases, kinases, transcription factors, ion channels and receptors. Furthermore, actions of dopamine vary greatly depending on target cell types, their activity states, strength and duration of receptor stimulation as well as other neuromodulators tapping into the same pathways (Tritsch and Sabatini, 2012).

Instead of directly exciting or inhibiting a target cell, dopamine usually modulates neurotransmission through other synapses (Figure 2.2). This neuromodulation influences the excitability of pre- and postsynaptic membranes, the amount of neurotransmitter released as well as receptor trafficking and sensitivity (Tritsch and Sabatini, 2012). Thus, dopamine either facilitates or hampers the information flow in neural circuits. This effect can be either transient or long lasting. Dopamine's effects can also be indirect. For example, (typically negative) modulation of transmitter release probability happens both directly via presynaptic, inhibitory D2 receptors and indirectly via postsynaptic retrograde mediators like hydrogen peroxide (H_2O_2), nitric oxide (NO) or endocannabinoids. D2 receptors are also located on dopaminergic terminals as autoreceptors providing negative feedback inhibition of dopamine synthesis and release (Ford, 2014). Figure 2.2 summarizes dopamine's potential modulatory effects on synaptic transmission.

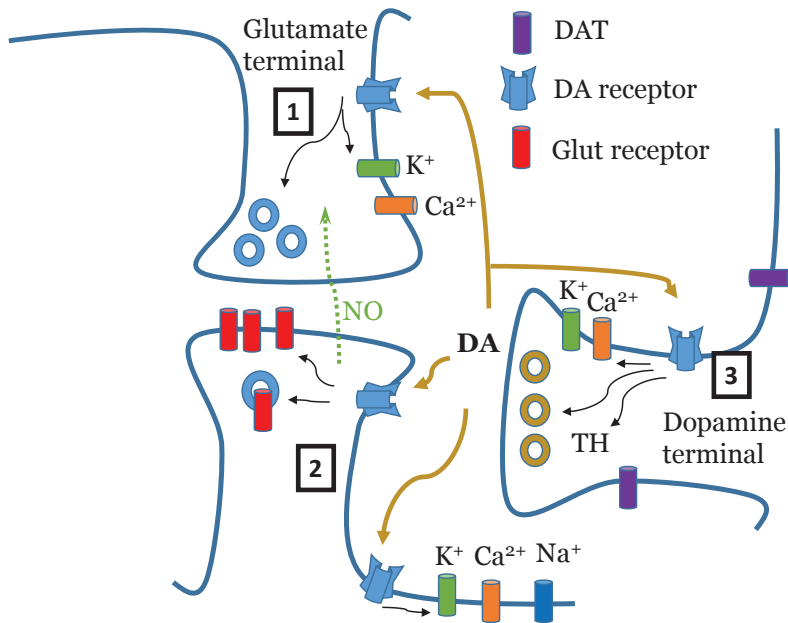


Figure 2.2. Dopaminergic modulation of synaptic transmission. Dopamine neurons do not usually form classical synapses with target cells, but modulate neurotransmission in other synapses. Dopamine's effects on presynaptic (1), postsynaptic (2) and dopaminergic (3) terminals are displayed. Dopamine transporters (DAT) are unable to prevent dopamine (DA) from escaping the site of release and activating receptors far away. Dopamine can modulate membrane excitability and transmitter release by its effects on ion channels (1-3) and vesicular release mechanisms (1, 3). These effects can be direct (1, 3) or indirect involving retrograde mediators from postsynaptic cells, like nitric oxide (NO) (2). Postsynaptic dopamine receptors influence signal detection by regulating receptor function and trafficking (2). Dopamine also mediates its own synthesis by tyrosine hydroxylase (TH) and release through D2 autoreceptors (3). Glut, glutamate. Figure inspired by (Tritsch and Sabatini, 2012) and (Rice and Cragg, 2008).

2.1.5 Dopaminergic modulation of basal ganglia output

Basal ganglia are a highly conserved chain of subcortical nuclei that play a key role in action selection and movement control. Their function is to select which one of the various competing neural input systems will receive access to motor mechanisms capable of driving behavior (Redgrave et al., 2011). Movements occur during pauses in the tonic inhibitory activity in the basal ganglia interface as specific voluntary motor programs are facilitated and the potentially interfering surrounding patterns are inhibited (Mink, 2003; Cisek and Kalaska, 2010). Two parallel pathways within the basal ganglia achieve this movement specificity together: The direct pathway facilitates the wanted movement patterns, while the indirect pathway suppresses the surrounding unwanted patterns (DeLong and Wichmann, 2007). Figure 2.3 depicts the basic organization of basal ganglia.

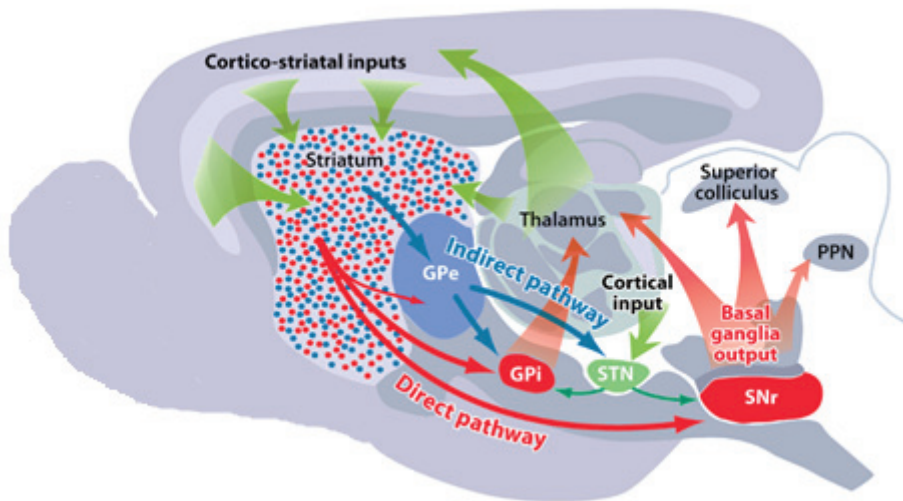


Figure 2.3. The basic organization of the basal ganglia circuits. The striatum receives excitatory glutamatergic input (wide green arrows) from cortex and thalamus. The inhibitory basal ganglia output (wide red arrows) projects from the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr) to thalamus, superior colliculus and pedunculo pontine nucleus (PPN). The direct pathway from D1 receptor expressing spiny projection neurons (SPNs; thin red arrows) projects directly to the output nuclei. The indirect pathway from D2 receptor expressing SPNs (thin blue arrows) projects only to the external segment of the globus pallidus (GPe), which together with the subthalamic nucleus (STN) connects the signal to the output nuclei. Unlike the rest of the basal ganglia nuclei, projections sent by STN are glutamatergic (thin green arrows). Figure adapted from (Gerfen and Surmeier, 2011).

Basal ganglia include the striatum, internal and external segment of globus pallidus (GPi and GPe), subthalamic nucleus (STN) and substantia nigra pars reticulata (SNpr). GPi and SNpr are the two basal ganglia output nuclei: GPi controls axial and limb movements and SNpr controls head and eye movements (Gerfen and Surmeier, 2011). The output nuclei project to thalamus, superior colliculus, and pedunculo pontine nucleus (PPN) (Figure 2.3) (Cisek and Kalaska, 2010; Gerfen and Surmeier, 2011). Striatum, which comprises dorsal striatum (or caudate putamen) and ventral striatum, is the largest of the basal ganglia nuclei. It is the principal integrator for basal ganglia information as it receives excitatory glutamatergic input from the cortical areas, limbic structures and thalamus (Sesack and Grace, 2010; Gerfen and Surmeier, 2011; Stuber et al., 2012). Striatum is believed to perform computation on sensorimotor, cognitive and emotional/motivational information to facilitate the selection of appropriate action (Cisek and Kalaska, 2010; Redgrave et al., 2011). Striatum also has the richest dopaminergic innervation in the entire central nervous system and dopamine can potentially modulate any information arriving there.

Striatum is almost fully populated by two types of GABAergic spiny projection neurons (SPNs; also called medium spiny neurons, MSN) that are nearly equal in numbers and constitute over 90 % of striatal cells (Gerfen and Surmeier, 2011). They form two parallel pathways from striatum to the two basal ganglia output nuclei: GPi and SNpr. The first group of GABAergic neurons express D1 receptors, substance P and dynorphin and projects directly to the output

nuclei (the striatonigral or so-called direct pathway; dSPNs) together with a minor axon collaterals to the GPe. The second GABAergic group express D2 receptors and enkephalin and projects exclusively to the GPe. GPe GABAergic neurons then project to the STN and to the output nuclei. Finally, STN glutamatergic neurons also project to the output nuclei forming a parallel pathway. This second pathway is called indirect pathway (iSPNs) (Gerfen and Surmeier, 2011). Figure 2.3 displays these complex striatal projections.

If there were not any inherent activity in the basal ganglia circuit, the inhibitory signal from the SPNs would simply silence the circuit further. However, as all the neurons in GPe, STN, GPi and SNpr are generating action potentials on their own (autonomous pacemakers), the GABAergic activity of SPNs is able to modulate the basal ganglia circuit output bidirectionally (increasing or decreasing) (Gerfen and Surmeier, 2011). The indirect pathway inhibits movement through the basal ganglia circuit, as it increases the inhibitory tone at the output interface. The direct pathway influence is facilitative as it causes a transient pause in the inhibitory tone, which allows specific movements to happen. As dSPNs express the facilitatory D1 receptors and iSPNs express the inhibitory D2 receptors, a phasic dopamine signal in the striatum has an opposite effect on the activity of these neurons. Hence, dopamine produces a transient motor signal by enhancing the direct pathway responsiveness and decreasing the opposing indirect pathway. This suggests a role for dopamine in motor signal gating.

The above model for dopaminergic regulation of striatal output is still relatively simple and straightforward. However, the actual situation is more complex. Next to the SPNs which make up the majority of the striatal neurons, the local striatal interneurons that also express dopamine receptors are important regulators of striatal circuits (Gerfen and Surmeier, 2011). These neurons constitute around 5 % of all neurons in the rodent striatum (Tepper et al., 2010). Currently the behavioral relevance of the local striatal interneurons remains very poorly understood, although they strongly influence basal ganglia output. There are three well characterized subtypes of GABAergic interneurons out of which parvalbumin expressing (PV⁺) and somatostatin, nitric oxide synthase and neuropeptide Y expressing (SOM/NOS/NPY⁺) neurons are described briefly below. For a comprehensive review see (Tepper et al., 2010). Cortical pyramidal neurons send glutamatergic projections directly to fast-spiking PV⁺ GABAergic interneurons in the striatum. The PV⁺ interneurons convey this activity to both direct and indirect SPNs eliciting inhibition (Tepper et al., 2010). This powerful feedforward inhibition is believed to contribute to action selection as it suppresses SPN activity in circuits associated with unwanted actions. In addition, PV⁺ interneurons receive inhibitory feedback projections from GPe neurons. Similarly, SOM/NOS/NPY⁺ GABAergic interneurons also form a similar corticostriatal feedforward circuit as PV⁺ interneurons, but this system is less well studied. Their ability to produce NO is believed to mediate important biochemical cross-talk between the striatal neurons (Calabresi et al., 2014).

In addition to the GABAergic interneurons, there is also one population of cholinergic interneurons in the striatum. Quite similar to the GABAergic interneurons, another major glutamatergic projection to the striatum comes from thalamus and connects to SPNs as well as the cholinergic interneurons. The cholinergic interneurons create another feedforward connection to SPNs that is biphasic. The first phase is inhibitory while the slower second one is excitatory and together they are thought to signal responses to salient stimuli. As the cholinergic interneurons carry dopamine receptors, dopamine is able to modulate this system as well. Moreover, in the striatum dopaminergic and cholinergic systems dynamically and reciprocally regulate each other in multiple different ways that are not completely resolved (Tritsch and Sabatini, 2012). A recent study showed how cholinergic interneurons can directly trigger dopamine release from

presynaptic terminals, bypassing dopamine neuron firing activity (Threlfell et al., 2012). Finally, the cholinergic interneurons have also been hypothesized to mediate the synaptic cross-talk between the two classes of SPNs (Calabresi et al., 2014). It was recently shown that striatal PV+ interneurons, although tightly interconnected with each other and SPNs, do not synapse with the cholinergic interneurons and only very weakly synapse with SOM/NOS/NPY⁺ interneurons (Szydlowski et al., 2013). This suggests independent roles for the different striatal interneuron populations in regulation of striatal output.

Another mechanism that complicates the classical model of dopamine regulation of basal ganglia function is related to learning. In addition to modulating the ongoing activity in the basal ganglia network (motor coordination), dopamine also regulates long-term changes in synaptic strength (Gerfen and Surmeier, 2011). These synaptic plasticity mechanisms include long-term depression (LTD) as well as long-term potentiation (LTP) and they are believed to underlie various aspects of learning and habit formation. LTD and LTP indicate persistent weakening or strengthening in synaptic transmission strength, respectively, based on recent activity patterns. In the striatum they provide a mechanism for the dopamine signal's ability to direct behavior towards rewarding cues and away from the aversive cues (Gerfen and Surmeier, 2011). Hence, a key role for dopaminergic basal ganglia regulation is the reinforcement of behaviors that have previously led to positive outcomes.

In conclusion, basal ganglia constitute a complex system of multiple interacting pathways. Dopamine modulates basal ganglia output through the prominent striatal input both directly and indirectly by modulating SPN activity and the various striatal interneurons. Importantly, dopamine modulates both immediate and long-term responsiveness of the system. Furthermore, the interneurons and other inputs also influence the striatal dopamine release as described above.

2.1.6 *Parkinson's disease and dopaminergic degeneration*

Indian medical literature described a neurological disease with slowness and akinesia (later known as *Kampavata*) as early as 600 BC and the condition was treated with powdered seeds of *atmagupta* (*Mucuna pruriens*) (Ovallath and Deepa, 2013). These seeds have been shown to contain 4-6 % of levodopa (Daxenbichler et al., 1972). In Western medical literature, James Parkinson provided the first coherent picture of PD symptoms in "An essay on the Shaking Palsy" in 1817 (Parkinson, 2002). However, it took nearly 150 years more before the dopamine-deficiency was associated to PD and the effectiveness of levodopa was demonstrated and brought into clinical practice (Degkwitz et al., 1960; Ehringer and Hornykiewicz, 1960; Birkmayer and Hornykiewicz, 1961; Cotzias, 1968).

PD is a chronically progressive neurodegenerative disease with strongly age-related prevalence. The classical motor symptoms of PD, including rigidity, bradykinesia and resting tremors, mostly result from the gradual degeneration and death of SNpc dopaminergic neurons and the consequent loss of dopamine in the dorsal striatum (Lees et al., 2009). Along with dopamine, various other neuronal systems also progressively degenerate in PD causing the different 'non-motor' symptoms of the disease including dementia, depression, sleep abnormalities, loss of smell and autonomic failure that manifests as constipation, incontinence and orthostatic hypotension (Meissner et al., 2011). The shared hallmark of the disease is the appearance of intracellular Lewy body inclusions that contain aggregated proteins, the most abundant being α -synuclein that normally resides in the nerve terminals (Goedert et al., 2013). Braak and coworkers originally identified that in sporadic PD the spread of brain Lewy

body pathology typically follows a specific ascending pattern from the brain stem towards the cortical areas (Braak et al., 2003). Few years later they extended their hypothesis with evidence suggesting that the Lewy pathology spread may transfer from the enteric nervous system (ENS) to the CNS via vagus nerve (Braak et al., 2006). Indeed, the newest evidence supports the view that Lewy pathology may transmit via neuronal synapses from ENS through the vagus nerve to and from the olfactory bulb to the SN and further areas of the brain (reviewed by Klingelhofer and Reichmann, 2015). Remarkably, the proposed initiation of PD pathology in the olfactory and gastrointestinal systems, the body's gateways to the environment, suggests high importance of environmental factors in PD pathogenesis.

Generally, the classical motor symptoms, and the concurrent diagnosis, of the disease appear when about 30 % of SNpc dopamine neurons and 50-60 % of striatal dopamine are lost (Burke and Malley, 2013). This suggests that the earliest PD pathology targets the enormous axonal tree of the dopamine neurons and the degeneration takes place through a "dying-back" axonopathy (Burke and Malley, 2013). This is further supported by the appearance of α -synuclein-positive aggregates in neurites prior to nerve cell bodies (Kanazawa et al., 2012). However, the dying-back theory also proposes that there is a clear window of opportunity to save and restore the degenerating axons in order to stop disease progression and alleviate the symptoms. The current therapies are entirely symptomatic aiming for replacement of striatal dopamine deficiency with the dopamine precursor levodopa (L-dopa), MAO-B and COMT inhibitors as well as with dopamine receptor agonists (Meissner et al., 2011). The problems include limited efficiency, motor state fluctuations, adverse side effects and inability of the therapies to slow down or reverse the neurodegenerative processes underlying the disease (Meissner et al., 2011). Due to this situation, there is a great demand for novel disease-modifying treatments that could affect the disease progression. The specific mutations in rare familial PD cases have directed the current research focus towards pathological alterations in axonal transport, mitochondrial function, energy metabolism and oxidative stress, as well as protein degradation, misfolding, and aggregation (Meissner et al., 2011).

None of the current animal models of PD fully recapitulates the human condition, especially the progressive and widespread Lewy body pathology, and PD appears to be a syndrome that specifically affects man. Neurotoxin-based models of dopaminergic degeneration (Parkinsonism) are the most widely used and they relatively well reproduce the classical motor symptoms of PD. Neurotoxicity of 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is based on a combination of oxidative stress and mitochondrial respiratory dysfunction, but in both cases degeneration is rapid and there is typically no Lewy-body pathology (Duty and Jenner, 2011). Furthermore, as they are both specifically taken up to dopaminergic cells by DAT (Duty and Jenner, 2011), any alterations in DAT function affects their toxicity, which can be a confounding factor in some genetically modified animals. Systemic MPTP does not work in rats, but is particularly effective in primates and appears to produce some relevant non-motor symptoms as well (Duty and Jenner, 2011). Rotenone is similar, but less specific and a much debated neurotoxin with variable effects (Duty and Jenner, 2011). However, its chronic, gastric administration might carry some relevance to human PD pathogenesis (Klingelhofer and Reichmann, 2015). Proteasome inhibitors like lactacystin and epoximycin directly inhibit cellular protein degradation to induce aggregation and cell death, which carries some clear validity to PD pathogenesis (Duty and Jenner, 2011). Various transgenic mouse models have been created based on the mutations found in rare familial PD cases, including various α -synuclein mutant mice. However, these animals do not appear to display proper

loss of nigrostriatal neurons (Duty and Jenner, 2011; Meissner et al., 2011). Adeno-associated viral (AAV) vector-delivered α -synuclein has been reported to produce prominent dopamine neuron loss in rats (Kirik et al., 2002; Decressac et al., 2012b). However, this model has been difficult to replicate by other groups (personal communication with docent Mikko Airavaara and others). More recently, a transgenic α -synuclein overexpression mouse line created using bacterial artificial chromosome was reported to display age-dependent dopamine neuron loss (Janezic et al., 2013). It remains to be seen how accurately this mouse line replicates human PD pathology. In conclusion, major progress in PD treatment will probably require development of better animal models that would be more relevant to the human condition.

2.2 Neurotrophic factors

Compared to most other cell types in the body, neurons exhibit a rather unique step in their development process: massive programmed cell death, which reduces 20-80 % of the initial numbers within a neuronal population (Oppenheim, 1991). This usually occurs relatively late in the neuronal maturation process following the phenotypic expression of their specific characteristics, especially the projection of axons to the target tissue (Oppenheim, 1991).

According to the classical neurotrophic factor hypothesis, young neurons compete for trophic factors that are released from the target in limited amounts (Hamburger and Levi-Montalcini, 1949). Those neurons that manage to connect adequately with the target receive enough trophic support to survive, while others die by programmed cell death. The vital importance of target-derived neurotrophic support is well established for most peripheral neurons. However, in the CNS the situation appears to be more complex as neurotrophic factors can also be secreted by neighboring cells or a cell can produce trophic factors for itself (autocrine loop) (Landreth, 1999; Cerchia, 2006). Even though the first target-derived neurotrophic factor, nerve growth factor (NGF), was originally identified and later purified according to this survival function (Hamburger and Levi-Montalcini, 1949), neurotrophic factors have many other functions as well. They stimulate and guide axonal growth and synapse formation, support neuronal phenotype and functions, protect the neurons from degeneration and regulate neuroplasticity. Additionally, most neurotrophic factors also have important functions outside the nervous system. Structurally neurotrophic factors are small, often glycosylated, polypeptides that are secreted into the extracellular space where they can diffuse and bind to their specific receptors. Neurotrophic factor receptors are often receptor tyrosine kinases (RTKs) that have an extracellular ligand binding domain, span the cell membrane once and have the kinase domain inside the cell. After receptor binding, neurotrophic factors can be retro- or anterogradely transported over long distances (Altar and DiStefano, 1998; Reynolds et al., 2000). Due to their potent trophic effects, neurotrophic factors represent attractive drug development targets to support neuronal survival and function.

Four major classes comprise the family of neurotrophic factors: (i) Neurotrophins include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and -4 (NT-4); (ii) the GDNF family of ligands (GFLs); (iii) neurotrophic cytokines (neurokines); and (iv) the newest family of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF).

2.3 GDNF family of neurotrophic factors

GDNF family of neurotrophic factors consists of four closely related members: (i) GDNF, (ii) Neurturin (NRTN), (iii) Artemin (ARTN) and (iv) Persephin (PSPN). GDNF was originally purified on the basis of its ability to support the survival of embryonic midbrain dopamine neurons (Lin et al., 1993) and NRTN by its ability to support sympathetic neurons (Kotzbauer et al., 1996). ARTN and PSPN were later identified and isolated by database search and homology cloning (Baloh et al., 1998; Milbrandt et al., 1998). GDNF family ligands (GFLs) are distant members of transforming growth factor- β (TGF- β) superfamily carrying the typical conserved seven cysteine residues, and belong to the “cystine knot” proteins (Airaksinen and Saarma, 2002). They function as disulfide-bonded homodimers that are produced as precursors (prepro-form), and pro-GFLs are further processed before or after secretion into their mature, biologically active forms (Airaksinen and Saarma, 2002; Lonka-Nevalaita et al., 2010). GDNF is expressed in two forms that are generated by alternative splicing and contain different pro-domains, (α)pro-GDNF and (β)pro-GDNF (Suter-Crazzolara and Unsicker, 1994; Grimm et al., 1998). Secretion of the (β)pro-GDNF appears to be activity-dependent, while (α)pro-GDNF is secreted by the constitutive pathway (Lonka-Nevalaita et al., 2010).

2.3.1 GDNF family receptors and signaling

All four GFLs first bind to their specific co-receptors, GDNF family receptor- α (GFR α) 1-4, that do not span the plasma membrane, but are either attached to it by a glycosyl phosphatidylinositol (GPI) anchor or after cleavage of the GPI-anchor exist in a soluble form (Airaksinen and Saarma, 2002) (Figure 2.4). GDNF, ARTN and NRTN can also bind heparin sulfates in extracellular matrix (ECM) and cell membrane with high affinity (Bespalov et al., 2011). This limits their diffusion and distribution and may accumulate, store and immobilize them in high concentrations to certain locations, which likely has important implications for receptor binding and signaling. GDNF preferentially binds to GFR α 1, NRTN to GFR α 2, ARTN to GFR α 3 and PSPN to GFR α 4, but there is also significant cross-reactivity between the GFLs and their co-receptors, although its physiological relevance is unclear (Figure 2.4). ARTN and PSPN co-receptors have not been found in the CNS and they presumably function only in the periphery. After GFL binding to their specific co-receptors the complex binds to and activates the common signaling receptor tyrosine kinase Ret (rearranged during transfection) (Durbec et al., 1996; Jing et al., 1996; Trupp et al., 1996). Ligand binding causes homodimerization of two Ret molecules, their reciprocal trans-autophosphorylation of certain Ret intracellular tyrosine residues, and finally activation of signaling cascades (Airaksinen and Saarma, 2002). As a typical receptor tyrosine kinase, Ret spans through the cell membrane only once and has the enzymatically active kinase domain inside the cell (Figure 2.4). The phosphorylated tyrosine residues of Ret serve as docking sites for adapter proteins and enzymes that activate specific downstream cascades. Such signaling cascades include for example: phosphatidylinositol-3-kinase (PI3K)/Akt, phospholipase C- γ (PLC γ)/protein kinase C (PKC), Src kinase and Ras/extracellular-signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK) pathways (Kramer and Liss, 2015).

Lipid rafts are ordered cell membrane microdomains that are enriched in sphingolipids, cholesterol and specific protein types, including GPI-anchored proteins (Simons and Sampaio, 2011). Its GPI-anchor localizes GFR α 1 to the lipid rafts (Figure 2.4), but Ret is excluded under

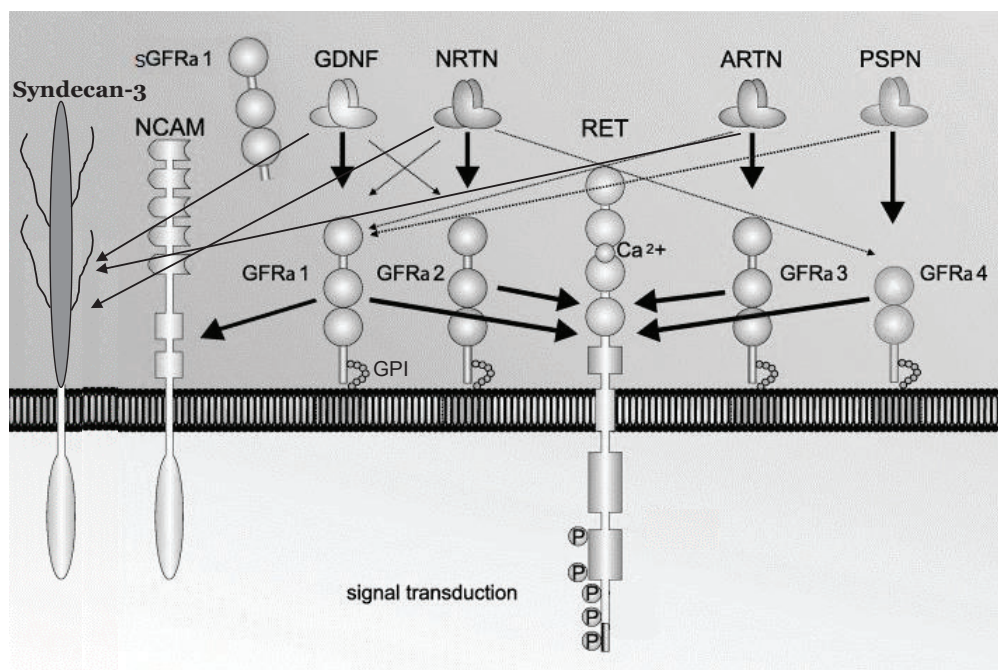


Figure 2.4. The GDNF family of ligands and their receptors. Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) bind preferentially their specific co-receptors GDNF family receptor α (GFR α) 1-4, although also significant cross reactivity exists (thin arrows). After that, the complex signals through the common receptor tyrosine kinase Ret or neural cell adhesion molecule (NCAM; GDNF only). GDNF can also bind to and signal through syndecan-3. Glycosyl phosphatidylinositol (GPI) anchor attaches GFR α receptors to the cell membrane, but the receptor also exists in a soluble form (sGFR α 1). Figure modified from Kramer & Liss 2015.

basal non-activated conditions (Tansey et al., 2000; Paratcha et al., 2001; Pierchala et al., 2006). In vitro GDNF and GFR α 1 together recruit Ret into the lipid rafts, which is important for subsequent downstream signal transduction (Tansey et al., 2000; Paratcha et al., 2001). The key importance of this mechanism for GDNF's physiological functions was very recently demonstrated in vivo with a knock-in mouse model, where GFR α 1 GPI anchor was replaced by a transmembrane domain that made GFR α 1 unable to translocate to the lipid rafts (Tsui et al., 2015). However, the transmembrane domain undoubtedly also inhibited the formation of soluble GFR α 1, which can activate Ret outside the rafts (Airaksinen and Saarma, 2002). Therefore, these novel mouse data are not specific enough to draw final conclusions regarding the importance of lipid raft localization. Furthermore, Ret and GFR α receptors may be co-localized in the same cell or expressed separately in projecting and target cells for signaling (Yu et al., 1998). This means that Ret can also interact with a GFR α receptor located in a separate cell ("in trans").

Throughout the brain GFR α s are much more widely expressed than Ret (Trupp et al., 1997; Yu et al., 1998), which suggests involvement of other Ret-independent signaling mechanisms. Indeed, GDNF/GFR α 1 complex is reported to also signal through neural cell adhesion molecule (NCAM) (Paratcha et al., 2003) to activate focal cell adhesion kinase (FAK) and Fyn, which control neuronal migration and synapse formation (Paratcha and Ledda, 2008). GDNF signaling

is also shown to involve the heparan sulfate proteoglycan, syndecan-3 (Bespalov et al., 2011). In addition to direct intracellular signal activation, syndecan-3 might also act as a co-receptor that concentrates and presents GDNF molecules to Ret and GFR α 1 (Bespalov et al., 2011). However, as Ret-deficient midbrain dopamine neurons fail to respond to GDNF or NRTN, it is likely that Ret is the principal signaling receptor for these neurons (Taraviras et al., 1999). Further evidence from studies with Ret-transgenic mice, reviewed below, support this view.

2.3.2 Neuronal effects of exogenous GDNF

According to earlier studies, dissociated embryonic dopamine neurons cultured with target cells (from striatum or PFC) grow target neuron-specific axons similarly to the *in vivo* target innervation (Hemmendinger et al., 1981). Culturing with striatal target cells also enhanced the phenotypic development, function and survival of dissociated dopamine neurons (Kotake et al., 1982; Hoffmann et al., 1983; Shalaby et al., 1983). These studies suggest that the target cells secrete a trophic signaling molecule for dopaminergic neurons. Subsequently, conditioned media derived from rat B49 glial cell line, established almost 20 years earlier (Schubert et al., 1974), was shown to promote the survival and dopamine uptake of cultured embryonic dopamine neurons (Engle et al., 1991). Soon, GDNF was purified and cloned at the biotechnology company Synergen from the same B49 glial cell line medium based on its ability to specifically support the survival and dopamine uptake of cultured dopaminergic neurons (Lin et al., 1993). In addition, recombinant GDNF increased neurite outgrowth and cell body size in these cultured neurons.

Remarkably, GDNF's potential to treat dopaminergic neurodegeneration, characteristic of PD, was already suggested in this initial report. This notion was soon supported by the demonstration of its neurorestorative properties in a neurotoxin-based rat model of PD (Hoffer et al., 1994). Since then the neuroprotective and neurorestorative properties of GDNF therapy have been solidly established in various delivery paradigms and models of dopaminergic degeneration in rodents and primates (Beck et al., 1995; Sauer et al., 1995; Tomac et al., 1995a; Shults et al., 1996; Cass, 1996; Gash et al., 1996, 2005; Bilang-Bleuel et al., 1997; Mandel et al., 1997; Tseng et al., 1997; Choi-Lundberg et al., 1997; Kearns et al., 1997; Rosenblad et al., 1998, 2000; Kirik et al., 2000b; Kordower et al., 2000; Kirik et al., 2000a; Georgievska et al., 2002b; Grondin et al., 2002). Importantly, Kirik and coworkers showed that striatal administration of GDNF provides higher functional benefits compared to nigral delivery in 6-OHDA lesioned rats (Kirik et al., 2000a), which is in line with the axonal die-back hypothesis (Burke and Malley, 2013). More recently it was shown that in a severe 6-OHDA lesion model the neurorestorative effect of GDNF is very modest (Voutilainen et al., 2011). Also, the failure of GDNF to provide neuroprotection in viral vector-mediated α -synuclein overexpression model of PD raised concerns on its efficiency in human PD (Lo Bianco et al., 2004; Decressac et al., 2011). However, it was subsequently demonstrated that this failure was due to downregulation of Nurr1 and consequently Ret by very strongly overexpressed α -synuclein and when Ret expression was restored by Nurr1 delivery, GDNF was effective in this model as well (Decressac et al., 2012a). It remains to be seen whether similar Ret and Nurr1 downregulation also happens in human PD and whether it contributes to the disease progression.

Exogenous GDNF has been shown to also support the survival of motoneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), peripheral sympathetic neurons (Ebendal et al., 1995), noradrenergic neurons (Arenas et al., 1995), parasympathetic neurons (Buj-Bello

et al., 1995), sensory neurons (Buj-Bello et al., 1995; Matheson et al., 1997), Purkinje cells from cerebellum (Mount et al., 1995) and basal forebrain cholinergic neurons (Williams et al., 1996).

An important part of the neuroprotective and restorative properties of GDNF is undoubtedly its ability to inhibit apoptosis, as was shown in postnatal cultured dopaminergic neurons (Burke et al., 1998). Remarkably, GDNF was the only trophic factor among the nine tested in the study that was able to support the survival of postnatal dopamine neurons *in vitro*, even though the other factors had previously shown effects in embryonic cultures (Burke et al., 1998). In support of its anti-apoptotic properties, striatal GDNF delivery suppressed postnatal apoptotic cell death of nigrostriatal dopamine neurons during the first, but not the second phase of natural developmental cell death of these neurons (Oo et al., 2003). Furthermore, GDNF neutralizing antibodies had an opposite effect in this study (Oo et al., 2003). Ectopic striatal transgenic overexpression of GDNF also increased the number of nigrostriatal dopamine neurons surviving the first phase of developmental cell death, but these changes did not persist to adulthood (Kholodilov et al., 2004). Using a viral vector approach the group provided further evidence that PI3K/Akt signaling would mediate the anti-apoptotic effects of exogenous GDNF during the natural cell death period of dopamine neurons (Ries et al., 2009). How endogenous GDNF regulates the developmental survival of dopamine neurons is currently not clear.

Injected GDNF protein is retrogradely transported from the striatum to the dopaminergic cell bodies that reside in the SNpc (Tomic et al., 1995b). Similarly, after gene delivery by a viral vector GDNF protein is transported to the SNpc (Georgievska et al., 2002b). This allows striatal delivery of GDNF to have specific effects also in the cell body that is located too far away for GDNF to reach via mere diffusion.

In addition to the robust trophic actions on injured dopamine neurons, GDNF delivery appears to have profound effects on intact midbrain dopamine systems. Nigral GDNF injection in adult rats increased spontaneous activity and decreased food consumption (Hudson et al., 1995). These behavioral effects were associated with increased nigral, but reduced striatal, dopamine levels and increased dopamine turnover in both compartments (Hudson et al., 1995). These findings were replicated by others in rats (Hebert et al., 1996; Martin et al., 1996) and largely in monkeys (Gash et al., 1995), but were also reported not to replicate in adult rats by one group (Beck et al., 1996). In addition, amphetamine-induced locomotor activity (Hudson et al., 1995) and dopamine release (Hebert et al., 1996) were increased in rats after nigral GDNF delivery. Similarly, striatal dopamine release was augmented after K⁺ stimulation in rats (Hebert et al., 1996) and monkeys (Gash et al., 1995) after nigral GDNF delivery. Chronic ventricular GDNF delivery in aged (over 20 years old) monkeys increased K⁺ and amphetamine stimulated dopamine release and improved hand motor functions (Grondin et al., 2003). Together these results overall suggest that ectopic GDNF enhances dopamine signaling in the nigrostriatal system. This is further supported by the findings that GDNF exposure *in vitro* increased vesicular dopamine content by 380 % and consequently the amount of dopamine released (Pothos et al., 1998). In addition, GDNF acutely inhibits A-type K⁺ channels in the midbrain dopamine neurons through the MAPK pathway leading to enhanced excitability of these neurons (Yang et al., 2001). GDNF also promotes formation of new functional dopaminergic axon terminals (Bourque and Trudeau, 2000).

Many of the reported effects on dopamine transmission are closely related to GDNF's ability to regulate dopamine synthesis through TH. Two-week GDNF infusion to the SN transiently reduced TH immunoreactivity in non-lesioned rats (Lu and Hagg, 1997). GDNF injection (10 µg) to the SN/VTA region caused 30 % and 60 % reductions in TH gene expression and mRNA

levels in VTA and SN, respectively (Messer et al., 1999). Additionally, striatal 13 months-long viral vector-mediated overexpression of GDNF up to 100 fold over the basal levels reduced cellular TH mRNA by 70 % and 40 % in the SNpc and VTA, respectively (Rosenblad et al., 2003). TH immunoreactivity in the striatum was also reduced up to 50 %, while dopaminergic terminals appeared to be unchanged (Rosenblad et al., 2003). This suggests that in an intact striatum GDNF does not cause aberrant sprouting, where dopaminergic neurites grow towards the GDNF source, unlike what happens after treating neurotoxin-induced damage (Kirik et al., 2000a, 2000b; Georgievska et al., 2002a). It has been hypothesized that the TH downregulation might be part of a regenerative response initiated by GDNF, as injured neurons switch their protein synthesis from functional to regenerative molecules (Grafstein, 1975).

However, it may be more likely that TH downregulation is a result of TH feedback inhibition by dopamine as GDNF, at least initially, enhances dopamine synthesis and release. TH downregulation may also be considered as a side effect of exogenous GDNF delivery. GDNF was shown to increase TH activity *in vitro* through phosphorylation of its serine 31 and 40 residues in rat and human cell lines (Kobori et al., 2004). This was confirmed *in vivo* in a study where striatal GDNF delivery in 24 month old rats reduced striatal TH levels but increased TH phosphorylation on serine 31, 40 and 19 residues (Salvatore et al., 2004). Nigral TH levels were unaltered, but serine 31 phosphorylation was specifically and strongly increased. These changes were associated with enhanced dopamine release after K⁺ and amphetamine stimulation in microdialysis experiments (Salvatore et al., 2004). However, it remains somewhat questionable which of the TH phosphorylation effects reported in these studies are direct and specific to GDNF and which ones indirect and secondary, especially as the GDNF dose utilized was very high (100 µg compared to 10 µg normally used in rats) (Salvatore et al., 2004). In support of this notion, many of the findings did not repeat similarly in a follow-up study and the lower GDNF dose (30 µg) only elevated serine 31 phosphorylation in the striatum (Salvatore et al., 2009).

As mentioned above, the initial study on GDNF reported its ability to stimulate dopamine uptake *in vitro* (Lin et al., 1993). However, the exact effects of exogenous GDNF on DAT function have remained obscure. It appears that GDNF's effects on DAT expression levels play only a minor role, as only a very high dose of striatally delivered GDNF (100 µg) reduced DAT levels, while a high dose of 30 µg had no effect (Salvatore et al., 2009). Similarly, viral delivery of GDNF to the dSTR was reported not to have effects on total DAT levels or its compartmental distribution but was reported to decrease dopamine uptake (Barroso-Chinea et al., 2016). This was associated with formation of DAT dimers and increased DAT interaction with α-synuclein (Barroso-Chinea et al., 2016). Recently, strong evidence was provided that endogenous GDNF through Ret reduces DAT activity by promoting its removal from the plasma membrane via direct interaction involving endocytosis mediator Vav2 (Zhu et al., 2015). Interestingly, this mechanism was reported to be specific for the ventral part of the striatum (Zhu et al., 2015). Hence, GDNF's effects on dopamine reuptake appear to be complex, region-specific and involve multiple mechanisms, but may also depend on GDNF source and dose. Clearly, more research is needed to uncover how GDNF influences striatal DAT function.

In conclusion, exogenous GDNF promotes dopamine neuron survival, growth and phenotype, which appear to underlie most of its functional effects in intact and lesioned animals. However, it is questionable whether these effects reflect the role of endogenous GDNF in the development, maintenance and function of midbrain dopamine neurons. Especially, the effects of huge doses of GDNF may be qualitatively very different from endogenous GDNF, whose expression levels and patterns are carefully regulated. Additionally, the commonly used *E. coli*

-produced GDNF may have different biological activity compared to mammalian GDNF due to folding issues and different post-translational modifications.

2.3.3 Endogenous GDNF and the midbrain dopamine systems

Despite being called “glial cell line-derived”, in the brain and in the striatum GDNF is not synthesized by glial cells. Early studies discovered that throughout the striatum GDNF mRNA is only expressed by diffusely scattered medium-sized neurons (Trupp et al., 1997; Oo et al., 2005). Only relatively recently it was discovered that nearly 95 % of GDNF expressing neurons in the mouse striatum are PV+ interneurons, the rest ~5 % being cholinergic or SOM+ interneurons (Hidalgo-Figueroa et al., 2012). Furthermore, it was shown that striatal GDNF expression peaks around P15 in mouse, being lower in young adults and much lower just after birth (Hidalgo-Figueroa et al., 2012). GDNF mRNA is prominently expressed in the nervous system throughout mouse embryonic development and in adulthood, while the expression of its receptors is more limited (Golden et al., 1999). The dramatic segregation of expression of GDNF and its receptors indicates that GDNF functions as an endogenous target-derived neurotrophic factor in the brain (Trupp et al., 1997; Golden et al., 1999). Highest GDNF expression in the adult brain is found in the striatum, nucleus accumbens, septum and thalamus (Trupp et al., 1997; Hidalgo-Figueroa et al., 2012). GDNF is also expressed in various non-neuronal organ systems in the body (Nosrat et al., 1996; Suvanto et al., 1996; Golden et al., 1999) and in general GDNF expression is much higher in the peripheral organs than in neuronal tissues (Suvanto et al., 1996; Trupp et al., 1997). This most likely reflects the critical role GDNF has in the correct development and function of those organs.

Mice completely lacking GDNF die within 24 hours after birth due to absence of kidneys and enteric neurons, but with intact midbrain dopamine systems (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). The same phenomenon also happens to the null mice for GFR α 1 (Cacalano et al., 1998; Enomoto et al., 1998) and Ret (Schuchardt et al., 1994), but not to the mice lacking other GFLs or their co-receptors (reviewed in Airaksinen & Saarma 2002). This indicates that GDNF/GFR α 1/Ret complex signaling has the key influence in the development of these peripheral organs. However, as the natural cell death period and maturation of midbrain dopamine neurons happens primarily after birth (Oo and Burke, 1997; Jackson-Lewis et al., 2000), the intact dopamine system in newborn GDNF null mice does not exclude the possibility that GDNF would have an important physiological role in the development and maintenance of these neurons.

The lethality of full GDNF knock-out mice still permits studies with heterozygous GDNF mice. Several groups have investigated the alterations in the dopamine systems of these mice that have only one functional GDNF allele. Gerlai and coworkers (2001) found no changes in striatal tissue levels of dopamine and DOPAC or in spontaneous or amphetamine-stimulated locomotion in 4-8 months old GDNF heterozygous mice, but reported an impairment in the water maze learning task (Gerlai et al., 2001). Similarly Airavaara et al. (2006) saw no differences in striatal tissue dopamine, DOPAC and HVA levels or in spontaneous or cocaine-stimulated locomotion in 3 months old GDNF heterozygous mice (Airavaara et al., 2006). However, they detected increased extracellular dopamine levels in striatum and nucleus accumbens and altered responses to morphine in these mice (Airavaara et al., 2004, 2006). In line with these findings, Heermann and colleagues (2010) found no changes in striatal dopamine, DOPAC and HVA levels, in SNpc TH+ cell numbers or in striatal TH+ optical density in 20-24 months old GDNF

heterozygous mice or even in GDNF/TGF β double heterozygous mice (Heermann et al., 2010). However, in striking contrast to the above studies Boger et al. (2007) reported increased striatal tissue levels of dopamine and DOPAC at 3 months of age, but reduced levels at 12 months of age together with diminished spontaneous locomotor activity in GDNF heterozygous mice (Boger et al., 2007). They also reported reduced TH+ SNpc cell number and striatal TH+ optical density along with reduced performance in accelerating rotarod in aged mice (Boger et al., 2006). Finally, they reported enhanced striatal dopamine uptake without changes in DAT levels, which may have contributed to the enhanced susceptibility to methamphetamine toxicity and enhanced nomifensine-induced locomotion in these mice (Boger et al., 2007; Littrell et al., 2012). In conclusion, the results from studies with GDNF heterozygous mice are highly controversial, but generally suggest subtle changes in dopamine neuron function, which may be age-related.

In addition to the above studies with GDNF heterozygous mice, one study reported a dramatic impact of a further 20 % reduction in adult GDNF expression down to 40 % of wild-type levels (Pascual et al., 2008). They generated GDNF conditional knock-out (cKO) or “floxed” mice, crossed these animals to Esr1-Cre transgenic line and injected tamoxifen at two months of age to activate Cre recombinase and hence to trigger GDNF deletion (Pascual et al., 2008). The resulting reduction in endogenous GDNF levels was reported to cause massive degeneration of SNpc and VTA dopaminergic neurons (60-70 % loss at 7 months after tamoxifen injections) and almost complete disappearance of brain noradrenergic neurons that reside in locus coeruleus (Pascual et al., 2008). The animals were also reported to develop hypokinesia (Pascual et al., 2008).

In the absence of follow-up studies with genetic GDNF models, Ret cKO mice have provided indirect *in vivo* evidence regarding GDNF's physiological role. Conditional deletion of Ret receptor from dopaminergic (DAT+) neurons caused no alterations in the number of TH+ cells in SNpc and VTA nor in TH+ optical density in the striatum or nucleus accumbens at 8-12 months of age (Jain et al., 2006). There were no differences in either striatal levels of dopamine or its metabolites or in behavioral tests measuring motor performance (Jain et al., 2006). The authors concluded that Ret is dispensable for the development and maintenance of midbrain dopamine neurons. However, another group that had followed similar mice until very old age (up to 24 months) detected late and progressive degeneration in the nigrostriatal dopaminergic system (Kramer et al., 2007). Their comprehensive characterization of these mice indicated that Ret-deficient dopaminergic fibers progressively degenerate in the striatum from 9 months onwards, their loss being most pronounced (over 50 %) at 24 months of age (Kramer et al., 2007). This was accompanied by similarly progressive loss of dopaminergic cell bodies in SNpc (no change at 3 months, ~25% loss at 12 and 38 % loss at 24 months), but not in the VTA (Kramer et al., 2007). Also, the noradrenergic neurons in the locus coeruleus remained intact when Ret was deleted from the entire CNS (Kramer et al., 2007). The degenerative changes were accompanied by reactive gliosis in the dorsal striatum and a neuroinflammatory response in the SNpc at 24 months, but with no changes in nigral α -synuclein levels (Kramer et al., 2007). Dopamine release and reuptake were both reduced at 12 and 24 months of age (Kramer et al., 2007). Surprisingly, the reported degenerative changes did not affect striatal levels of dopamine and DOPAC, spontaneous locomotor activity or motor performance in the accelerating rotarod (Kramer et al., 2007). A follow-up study found that these mice do not display increased sensitivity to the dopaminergic neurotoxin MPTP, but the recovery of their dopaminergic fibers in the striatum is impaired compared to the controls (Kowsky et al., 2007). Together these results suggest a role for

Ret signaling in the long-term maintenance of nigrostriatal dopamine system and in recovery of striatal dopaminergic axonal terminals after injury.

Further evidence about the significance of Ret signaling comes from studies with knock-in multiple endocrine neoplasia type B (MEN2B) mice, where a single point mutation (Met918Thr) renders the intracellular tyrosine kinase domain of Ret constitutively active (Takahashi, 2001). Although originally developed as a cancer model (Smith-Hicks et al., 2000), these mice were shown to have greatly increased levels of dopamine and its metabolites in dorsal and ventral striatum (up to 100 % elevation in homozygous mice), cortex and hypothalamus at 2-3 months of age (Mijatovic et al., 2007). This was accompanied by increased levels of striatal TH and DAT as well as by modestly increased numbers of TH+ neurons in SNpc (but not in VTA) and DAT+ terminals in the striatum (Mijatovic et al., 2007). Finally, their spontaneous locomotor activity was reduced, but cocaine-stimulated activity was augmented (Mijatovic et al., 2007). In follow-up studies these mice were shown to have increased dopamine synthesis, storage, release and reuptake in the striatum (Mijatovic et al., 2008) and they were shown to be protected against the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) (Mijatovic et al., 2011).

Contrarily to the Ret-deficient mice, the results from MEN2B mice suggest a potential role for Ret signaling in the development, function, protection and maintenance of the nigrostriatal dopaminergic system. There are several possible explanations for this discrepancy. Both the complete removal of Ret from dopaminergic neurons and the unnaturally strong and continuous Ret activation may trigger secondary, compensatory mechanisms that mask or confound the primary effects of the genetic manipulations. Furthermore, when drawing conclusions from these Ret mutants to endogenous GDNF it is important to note that all the GDNF family ligands signal through Ret, so the observed alterations can represent actions of separate ligands. GDNF is reported to also signal Ret-independently (Paratcha et al., 2003; Bespalov et al., 2011), so endogenous GDNF might have additional effects to those suggested by the Ret mutant mice. Hence, the current data regarding endogenous GDNF is incomplete, at least in part controversial and only partially in line with studies using exogenous GDNF. Novel animal models are needed to resolve GDNF's role as a regulator of midbrain dopamine neurons.

2.3.4 Exogenous GDNF and NRTN in human clinical trials

The promising preclinical results have taken GDNF protein to three phase 1 and two phase 2 clinical trials with human PD patients. NRTN has also been tested using gene therapy in one phase 1 and two phase 2 clinical trials based on its ability to protect and rescue dopamine neurons in animal models of PD (Oiwa et al., 2002; Kordower et al., 2006; Gasmi et al., 2007). As polypeptides, neurotrophic factors are quickly degraded after oral administration and do not cross the blood-brain barrier. Hence, they need to be delivered directly into the brain.

In the first phase 2 study GDNF protein was delivered to the lateral ventricle of advanced PD patients without achieving clinical benefit but rendering various systemic adverse effects (Nutt et al., 2003). It was subsequently found that GDNF failed to reach the target tissues putamen (striatum) and SN (Kordower et al., 1999; Nutt et al., 2003). Consequently, intracerebroventricular delivery route was abandoned.

To the contrary, direct 12-month long striatal delivery of GDNF in two phase 1 studies was found safe (Gill et al., 2003; Slevin et al., 2006). However, the subsequent double-blind, placebo-controlled trial was stopped prematurely at 6 months due to lack of efficacy (Lang et al., 2006). There is clear evidence that the different pump and catheter used in this study resulted in

insufficient distribution of GDNF throughout the putamen to provide clinical benefits (Salvatore et al., 2006; Morrison et al., 2007).

To improve the distribution of the trophic factor in the target region and achieve more convenient and sustained delivery, the recent focus has been in viral vector-mediated delivery. An open-label study with bilateral adeno-associated virus type 2 (AAV2) delivery of NRTN to putamen of 12 advanced PD patients reported very good safety and tolerability as well as improved motor function at 1 year (Marks et al., 2008). However, a double-blind, sham-surgery controlled 12-month phase 2 trial with 58 advanced PD patients showed no significant benefit compared to controls at 12 months, but the procedure itself was again well tolerated (Marks et al., 2010). However, the study showed modest, but significant benefits at 18 months for AAV2-NRTN treatment, although this sample of patients was much smaller (Marks et al., 2010). In addition, even though the striatal distribution of NRTN in the PD patients was comparable to that observed in similarly treated MPTP-lesioned monkeys, nigral NRTN expression was nearly absent in PD patients while in the monkey model it was prominent (Bartus et al., 2011, 2015). This suggests poor NRTN diffusion and impaired axonal transport of NRTN to the SNpc in the PD patients. To test this possibility an 18-month double-blind, sham-controlled trial with 51 advanced PD patients was conducted using higher AAV2-NRTN doses and bilateral delivery to both putamen and SNpc. However, this study did not report significant benefits compared to the sham surgery, even though the treatment was again safe and well tolerated (Olanow et al., 2015). Nevertheless, a subgroup of less advanced PD patients, diagnosed within 5 years prior to the treatment, appeared to respond better to the treatment compared to more advanced patients (Olanow et al., 2015).

In conclusion, so far no double-blind phase 2 trial with GDNF or NRTN has demonstrated clear clinical benefits to patients with advanced PD. There are many possible reasons for these failures, despite the repeated success in animal models. Impairment of axonal transportation and possible lack of anterograde transportation might hamper the movement of trophic factors from the striatum to the SNpc as well as the transmission of survival and growth signals from SNpc to the striatum (Olanow et al., 2015). Dopamine neurons in advanced PD patients may be too few or too dysfunctional to respond to the treatment (Kordower et al., 2013) and hence earlier disease stage patients should benefit more from the trophic factor therapy. Adequate delivery and diffusion of the trophic factors may pose a general problem in the large human brain compared to the smaller brains of rodents and rhesus monkeys. In addition, there is currently poor understanding of the roles that endogenous neurotrophic factors play in aging and diseased dopamine neurons. Additional clinical studies with trophic factors are still underway, including AAV2 gene delivery of GDNF and continuous GDNF infusion into the putamen (Olanow et al., 2015). However, better understanding and utilization of the general cell biology knowledge of GFLs will be most likely needed to improve therapeutic success of GDNF-based therapies, like small molecular weight GDNF-mimetics.

3 AIMS OF THE STUDY

Despite intensive research on the *in vivo* effects of exogenous GDNF, the role of endogenous GDNF in the development, maintenance and function of the midbrain dopamine neurons remains largely unknown. With the novel GDNF animal models, we aimed to clarify the role of physiological GDNF in the development, maintenance and function of the midbrain dopamine neurons.

The specific aims of the study were:

1. Investigate the role of endogenous GDNF in the maintenance of nigrostriatal dopaminergic system during development and early postnatal maturation (Studies I and III)
2. Clarify the role of endogenous GDNF in the maintenance of midbrain dopamine systems during aging (Studies I and III)
3. Characterize the effects of endogenous GDNF on dopamine levels, release and reuptake (Studies II and III)
4. Examine the ability of endogenous GDNF to protect nigrostriatal dopamine system from neurotoxic lesions (Study III)

4 MATERIALS AND MAIN METHODS

4.1 New GDNF animal models

4.1.1 GDNF conditional knock-out mice

In order to circumvent the problem of early lethality in mice without GDNF (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996), we used Cre-Lox recombination technology to develop a mouse strain where GDNF can be conditionally deleted to preserve its vital role in the development of peripheral organs. *Gdnf* exon 3, which encodes for the mature GDNF protein, was flanked with specific LoxP sequences that the virus-derived enzyme Cre can recognize (Study I, Figure 1a). This resulted in the *Gdnf* “floxed” mice. When Cre is expressed in a cell, it permanently cleaves away DNA between the two LoxP sites and rejoins the remaining DNA strands back together. Hence, by controlling Cre expression in the *Gdnf* floxed mice, we could specify where and when *Gdnf* is deleted.

GDNF exon 3 was conditionally deleted by three complementary approaches: (i) Crossing the floxed mice to transgenic Nestin-Cre line (Tronche et al., 1999; Kramer et al., 2007) results in removal of GDNF from the entire central nervous system (CNS) around E10. (ii) Unilateral injection of adeno-associated virus serotype 5 encoding Cre (AAV5-Cre) into dorsal striatum (dSTR) of 2 months old floxed mice removes GDNF specifically from this innervation target of nigrostriatal dopamine neurons. (iii) Crossing the floxed mice to *Esr1*-Cre line where Cre activity can be used upon tamoxifen (TMX) application (Hayashi and McMahon, 2002). Using a similar GDNF floxed allele and the same *Esr1*-Cre line, Pascual et al. reported that intraperitoneal (i.p.) tamoxifen (TMX) injections (200 mg/kg per day for four days) at two months of age cause a significant 60 % reduction in the brain GDNF levels (Pascual et al., 2008). To replicate Pascual et al. experiments, we used the same injection protocol (Pascual et al., 2008).

In the case of Nestin-Cre and *Esr1*-Cre lines we studied compound heterozygous mice with one regular knock-out allele (KO) and one floxed allele (F) for *Gdnf* as in (Kramer et al., 2007; Pascual et al., 2008). All the mouse lines (including GDNF hypermorphic mice below) were kept in a triple mixed 129Ola/ICR/C57bl6 genetic background. They were housed in 12/12 light/dark cycle with “lights-on” at 06:00, at ambient temperature of 20–22 °C, 2–5 animals per cage with *ad libitum* access to regular chow and tap water. All animal experiments were authorized by the National Animal Experiment Board of Finland.

4.1.2 GDNF hypermorphic mice

GDNF hypermorphic (GDNFh) mice represent a novel type of genetically modified animal model. In these mice, the physiological GDNF expression is enhanced, but the gene 5' regulatory region, exon and introns remain intact following the regulatory control of native promoters and enhancers. The endogenous 3' untranslated region (3'UTR) of *Gdnf* gene was replaced by a construct (puΔtk cassette; Study III, Figure 1) that renders the resulting mRNA largely unresponsive to the negative microRNA (miRNA) regulation that destabilizes the native *Gdnf* mRNA. Increased mRNA stability allows more of native GDNF protein to be produced everywhere in the body where and when GDNF is normally expressed. Hence, the normal temporal and spatial pattern of *Gdnf* expression is retained.

As the homozygous GDNFh animals die by two weeks of age due to kidney defects (Study III, Figure 2F-I) we conducted all our adult studies with heterozygous GDNFh mice.

4.2 Main methods

The main methods used in this study are listed in Table 4.1. Detailed descriptions of materials and methods can be found in the original publications and their supplements, which are referred to by their Roman numerals.

Table 4.1. Methods used.

Method	Used in	Author personally involved
Amphetamine-induced locomotion	II, III	II, III
Brain dissection	I, II, III	I, II, III
Corridor test	I, III	III
Determination of monoamines	I, II, III	I, II, III
Fast-scan cyclic voltammetry	II, III	
GDNF ELISA	I, III	
Immunohistochemistry	I, III	I, III
<i>In vivo</i> chronoamperometry	II, III	
<i>In vivo</i> microdialysis	II, III	II, III
Membrane protein biotinylation	II	
Optical densitometry analysis	I, III	I
Quantitative PCR	I, III	
Stereological analysis	I, III	I, III
Stereotactic surgery	I, II, III	II, III
Transcardiac perfusion	I, III	I, III
Western blotting	II, III	

5 RESULTS

5.1 Brain GDNF expression in GDNF conditional knock-out mice (Study I)

GDNF messenger RNA (mRNA) measurements revealed that GDNF expression was either absent or very low in the presence of Cre in all the ‘floxed’ mouse strains (Figure 5.1). In adult Nestin-Cre animals (F/KO + Nestin-Cre) GDNF mRNA was virtually absent in the dopaminergic brain regions investigated, while the heterozygous animals (C/KO) had only ~50 % reduction (Figure 5.1a). Intrastriatal injection of AAV5-Cre caused nearly full GDNF deletion in the injected striatum by 9 months after injection (Figure 5.1b). At the age of 5 months, i.e. 3 months after TMX injections Esr1-Cre mice had around 85 % reduction in the whole brain GDNF mRNA levels, but unexpectedly this appeared to happen independently of TMX (Figure 5.1c). At two months of age these animals still had normal GDNF mRNA levels (Study I, Figure 3b).

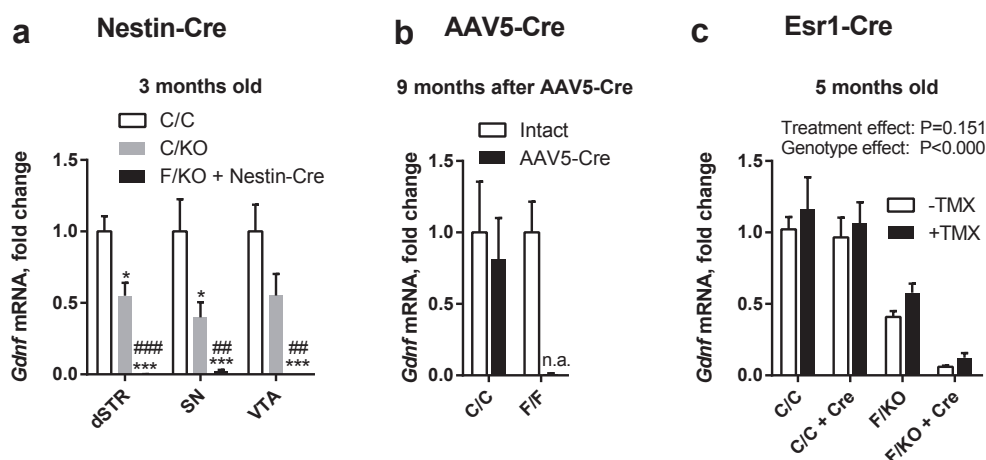


Figure 5.1. Relative levels of *Gdnf* mRNA in GDNF cKO mice. (a) *Gdnf* mRNA levels were halved in C/KO animals and were virtually absent in F/KO + Nestin-Cre in all the brain areas studied. (b) Striatal AAV5-Cre injection did not have an effect on C/C mice, but in F/F animals *Gdnf* mRNA was nearly absent in the injected striatum. (c) *Gdnf* mRNA levels were halved in F/KO brain and greatly reduced in F/KO + Cre. TMX injections did not have an effect on *Gdnf* expression levels. F-floxed; KO-knock-out; C-control containing both wild-type and floxed alleles, except for AAV5-Cre only wild-type alleles; dSTR-dorsal striatum; SN-substantia nigra; VTA-ventral tegmental area; n.a. = not applicable; TMX-tamoxifen; *, *** p < 0.05 or 0.001 relative to C/C, ##, ### p < 0.01 or 0.001 relative to C/KO. Kruskal-Wallis test with stepwise-stepdown multiple comparison and two-way ANOVA were used for statistical analysis. Error bars are mean \pm s.e.m.

5.2 TH-positive cell numbers in GDNF conditional knock-out mice (Study I)

Despite the removal or clear reduction of GDNF mRNA expression, stereological analysis of TH+ cells indicated no alterations in the numbers of dopaminergic neurons in the SNpc, even after aging (Figure 5.2). Nestin-Cre mice were studied at the ages of 3, 14 and 19 months (Figure 5.2a) and AAV5-Cre and Esr1-Cre mice at the age of 9 months; 7 months after their respective injections (Figure 5.2b and c). VTA was also analyzed in 19 months old Nestin-Cre mice without detecting significant differences (Figure 5.2a). Finally, TH+ optical density in dSTR and ventral striatum (vSTR) was unchanged in Nestin-Cre mice (Study I, Figure 1g).

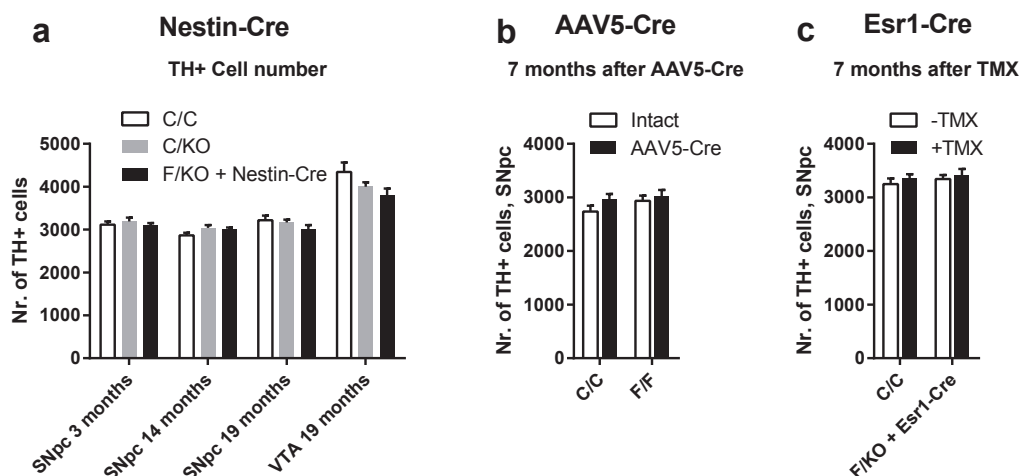


Figure 5.2. Midbrain TH+ cell numbers in GDNF cKO mice. (a) There were no differences in TH+ cell numbers between the genotypes in SNpc or VTA in Nestin-Cre mice. (b) There were no differences in SNpc TH+ cell numbers between the intact and AAV5-Cre injected side. (c) There were no differences between the groups in SNpc TH+ cell numbers in Esr1-Cre mice. TH-tyrosine hydroxylase; F-floxed; KO-knock-out; C-control containing both wild-type and floxed alleles, except for AAV5-Cre only wild-type alleles; SNpc-substantia nigra pars compacta; VTA-ventral tegmental area; TMX-tamoxifen. Error bars are mean \pm s.e.m.

5.3 Monoamine concentrations in different brain areas of GDNF conditional knock-out mice (Study I and II)

In line with the TH+ cell counts and striatal TH+ optical density analysis there were no differences in the brain tissue levels of dopamine or its metabolites DOPAC and HVA in Nestin-Cre GDNF cKO strain or in AAV5-Cre injected mice. Table 5.1 summarizes the average levels of these monoamines in dSTR, vSTR, SN) and VTA in Nestin-Cre line and in dSTR of AAV5-Cre injected mice. There were no differences in dopamine turnover $[(DOPAC+HVA)/dopamine]$ in any brain area (unpublished data).

Table 5.1 Cerebral tissue concentrations of dopamine, DOPAC and HVA (ng/g of wet tissue) in Nestin-Cre and AAV5-Cre lines.

Nestin-Cre	Dopamine	DOPAC	HVA
Dorsal striatum			
C/C	14 206±957	552±91	240±34
C/KO	14 598±1 099	485±59	395±105
C/KO + Nestin-Cre	14 476±1 020	422±72	502±143
Ventral striatum			
C/C	9 527±755	437±48	171±44
C/KO	9 276±594	394±34	121±30
C/KO + Nestin-Cre	9 690±514	409±40	173±35
Substantia Nigra			
C/C	253±25	80±11	59±9
C/KO	233±22	77±6	59±12
C/KO + Nestin-Cre	245±34	70±12	72±24
Ventral tegmental area			
C/C	598±39	189±20	136±40
C/KO	589±29	229±27	99±17
C/KO + Nestin-Cre	610±43	200±12	153±43
AAV5-Cre	Dopamine	DOPAC	HVA
Dorsal striatum			
C/C	10 756±910	820±98	348±93
C/C + AAV5-Cre	10 444±1 082	973±168	262±59
C/F	11 233±451	955±83	373±73
C/F + AAV5-Cre	11 304±599	781±78	345±68
F/F	10 719±850	791±83	401±83
F/F + AAV5-Cre	10 866±718	834±82	428±76
Average ± SEM			

5.4 Amphetamine effects on Nestin-Cre GDNF conditional knock-out mice (Study II)

Even though the numbers of TH+ cells and brain dopamine levels were unchanged in Nestin-Cre GDNF cKO mice that lack brain GDNF, these mice respond differently to amphetamine compared to their GDNF-expressing littermates (Figure 5.3). Amphetamine enters dopaminergic terminals via DAT, displaces dopamine from the storage vesicles and causes DAT-dependent dopamine efflux to the extracellular space (German et al., 2015). Amphetamine also directly inhibits DAT function, which contributes to the increased extracellular dopamine levels and the consequent psychomotor stimulation (German et al., 2015). Amphetamine-induced (1 mg/kg, i.p.) hyperlocomotion was blunted in the absence of brain GDNF (Figure 5.3a). As amphetamine response is unaltered in GDNF heterozygous mice (Gerlai et al., 2001), we compared F/KO + Nestin-Cre mice only to their C/KO littermates to have large enough groups. The blunted psychomotor stimulation appears to be due to reduced amphetamine-induced dopamine efflux through DAT, which was determined by cyclic voltammetry in striatal slices (Figure 5.3b). In the absence of brain GDNF striatal DAT seems more resilient to reverse from pumping dopamine into the cells to releasing the cytosolic dopamine into the extracellular space as happens in the presence of amphetamine (Figure 5.3c). The baseline open field activity was unchanged in these mice (Study I, Supplementary Table 1). Furthermore, amphetamine depleted synaptic vesicles similarly in the presence and absence of brain GDNF (Study II, Supplementary Figure 1a).

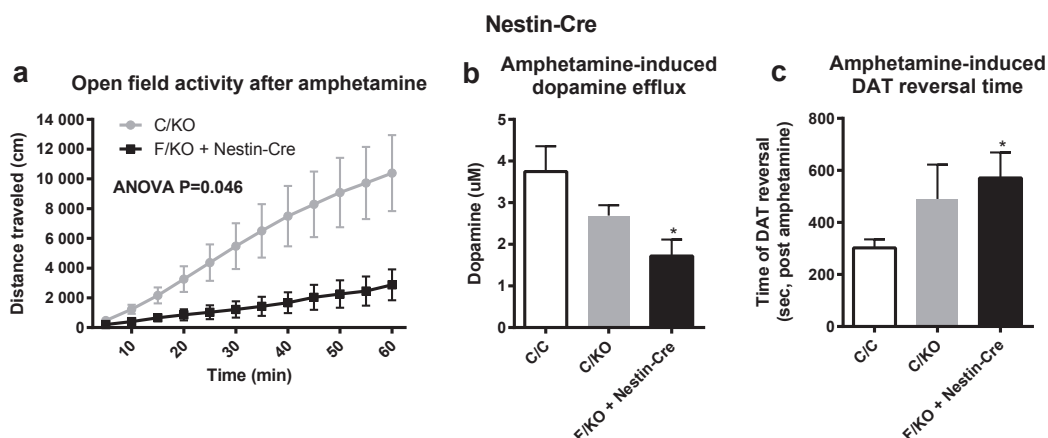


Figure 5.3. Amphetamine effects on Nestin-Cre GDNF cKO mice. (a) Open field locomotor activity after amphetamine injection (1 mg/kg) showed reduced hyperlocomotion in the absence of brain GDNF. (b) Dopamine efflux caused by amphetamine was significantly attenuated in striatal slices in the absence of brain GDNF. (c) In the absence of brain GDNF it took nearly twice as long for DAT to reverse the direction of dopamine flow after adding amphetamine on the striatal slices. F-floxed; KO-knock-out; C-control containing both wild-type and floxed alleles; DAT-dopamine transporter. *, $p < 0.05$ relative to C/C. One-way ANOVA for repeated measures and Kruskal-Wallis test followed by Mann-Whitney U post hoc tests and Bonferroni correction were used for statistical analysis. Error bars are mean \pm s.e.m.

5.5 Dopamine release and uptake in the absence of brain GDNF (Study II)

The interesting results with amphetamine encouraged us to investigate dopamine release and uptake in Nestin-Cre GDNF cKO mice. Single pulse -stimulated dopamine release was similar between the genotypes when measured by cyclic voltammetry in striatal slices (Figure 5.4a). However, dopamine uptake was augmented in the absence of brain GDNF as determined by chronoamperometric studies in anesthetized animals (Figure 5.4b). Cyclic voltammetry studies in striatal slices also supported this finding (Study II, Figure 2C-F). We found increased DAT tissue levels in the dSTR of Nestin-Cre GDNF cKO mice with a smaller increase in C/KO mice that lack one *Gdnf* allele (Figure 5.4c). Moreover, in dSTR synaptosomal preparations significantly higher proportion of DAT was located in the cell surface in mice lacking brain GDNF (Figure 5.4d). Hence, endogenous GDNF negatively regulates total DAT protein levels and DAT protein levels in synaptosomal membranes explaining the enhanced dopamine uptake in Nestin-Cre GDNF cKO mice.

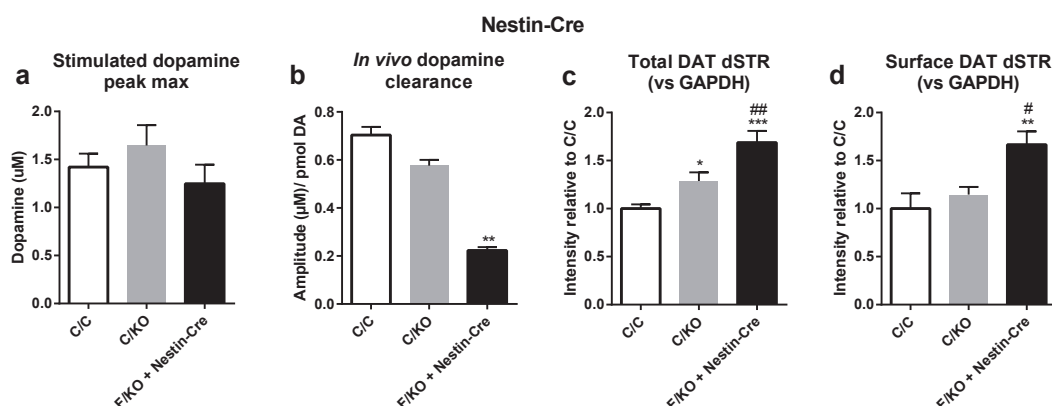


Figure 5.4. Dopamine release and uptake in Nestin-Cre GDNF cKO mice. (a) There were no differences in single pulse -stimulated dopamine release between the genotypes, when measured by cyclic voltammetry in striatal slices. (b) DAT activity, as determined by disappearance of dopamine applied to the striatum of anesthetized animals, was enhanced in the absence of brain GDNF. (c) Total tissue levels of DAT were clearly increased in dSTR of mice lacking brain GDNF, but also slightly increased in C/KO mice consistent with *Gdnf* gene dose. (d) Biotinylation analysis for surface expression of DAT in dSTR synaptosomal preparations indicated that higher proportion of DAT is located in the cell surface in F/KO + Nestin-Cre mice. F-floxed; KO-knock-out; C-control containing both wild-type and floxed alleles; DAT-dopamine transporter, GAPDH-glyceraldehyde 3-phosphate dehydrogenase. *, **, *** $p < 0.05$, 0.01 or 0.001 relative to C/C; #, ## $p < 0.05$ or 0.01 relative to C/KO. Kruskal-Wallis test followed by Mann-Whitney U post hoc tests and Bonferroni correction and one-way ANOVA followed by Student-Newman-Keuls test were used for statistical analysis. Error bars are mean \pm s.e.m.

5.6 Brain GDNF expression in GDNF hypermorphic mice (Study III)

In GDNF hypermorphic (GDNFh) mice *Gdnf* mRNA levels are increased in the brain (Figure 5.5) and elsewhere in the body (Study III, Figure 2C-E, S2C-D), wherever GDNF is natively expressed. Analysis of *Gdnf* mRNA expression suggests that the exact extent of increase is dependent on the brain area and the developmental state of the animal (Figure 5.5a, b). In dSTR *Gdnf* mRNA levels were approximately doubled in heterozygous and tripled in homozygous animals. In support of this, GDNF protein levels in the adult dSTR were increased to the same extent as the mRNA levels (Study III, Figure 3H).

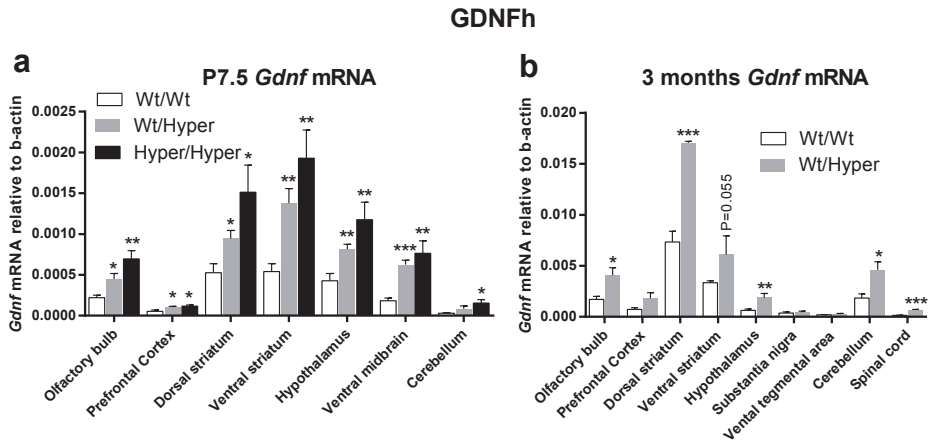


Figure 5.5. GDNF mRNA expression in GDNFh mice. (a) *Gdnf* mRNA levels at different brain areas of GDNFh mice at P7.5. (b) *Gdnf* mRNA levels at different brain areas of GDNFh adult mice. *, **, *** $p < 0.05$, 0.01 or 0.001 relative to Wt/Wt. One-way ANOVA followed by Student-Newman-Keuls test and Student's t-test were used for statistical analysis. Error bars are mean \pm s.e.m.

5.7 TH+ and VMAT2+ cells in substantia nigra and striatal DAT+ varicosities in GDNF hypermorphic mice (Study III)

In GDNFh mice enhanced *Gdnf* expression leads to ~15 % increased number of dopaminergic cells in SNpc as stereologically estimated by TH+ counting (Figure 5.6a) and confirmed by vesicular monoamine transporter 2 (VMAT2)+ counting (Figure 5.6b). The numbers of TH+ cells were increased already at P7.5 and remained increased at 3 months of age (Figure 5.6a). In dSTR enhanced GDNF expression led to increased number of DAT+ varicosities (Figure 5.6c) that represent dopaminergic terminals. DAT+ striatal area was unchanged (Study III, S3I).

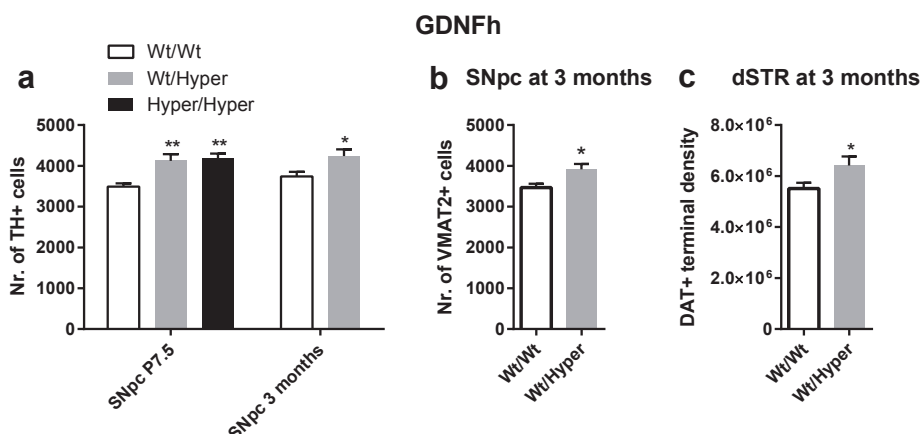


Figure 5.6. TH+ and VMAT2+ cell and DAT+ striatal terminal counts in GDNFh mice. (a) Unbiased stereological TH+ cell counts indicated higher numbers of dopaminergic cells in mice carrying *Gdnf* allele. The number of TH+ cells was increased already at P7.5 and remain increased in adulthood (3 months) and old age (17 months). (b) The number of VMAT2+ cells was increased at 3 months of age in GDNFh mice. (c) Unbiased stereological counts for DAT+ varicosities in dSTR revealed increased number of dopamine terminals in adult GDNFh mice. TH-tyrosine hydroxylase; VMAT2-vesicular monoamine transporter 2; DAT-dopamine transporter; SNpc-substantia nigra pars compacta; dSTR-dorsal striatum. *, ** $p < 0.05$ or 0.01 relative to Wt/Wt. One-way ANOVA followed by Student-Newman-Keuls test and Student's t-test were used for statistical analysis. Error bars are mean \pm s.e.m.

5.8 Monoamine concentrations in different brain areas of GDNF hypermorphic mice (Study III)

In line with the increased number of dopaminergic cells and striatal DAT+ terminals, tissue dopamine levels were found to be consistently ~25 % elevated in the dSTR of GDNFh mice (Table 5.2). This elevation was detected already at P7.5 rostral brain and also in adult GDNFh mice (Table 5.2). Furthermore, DOPAC levels were also elevated in dSTR (Table 5.2). In vSTR dopamine and DOPAC levels were not significantly elevated (Table 5.2). There were no differences in dopamine turnover [(DOPAC+HVA)/dopamine] in any brain area (unpublished data).

Table 5.2 Cerebral tissue concentrations of dopamine, DOPAC and HVA (ng/g of wet tissue) in GDNFh mice at different ages.

P7.5	Dopamine	DOPAC	HVA
Rostral brain			
Wt/Wt	517±22	41±5	19±4
Wt/Hyper	660±50*	53±8	23±6
Hyper/Hyper	623±31*	53±5	27±5
3 months	Dopamine	DOPAC	HVA
Dorsal striatum	***	**	P=0.057
Wt/Wt	14 603±525	371±31	143±22
Wt/Hyper	17 959±378	531±40	218±31
Ventral striatum			
Wt/Wt	9 664±281	326±24	61±12
Wt/Hyper	10 228±396	391±38	76±21

*, **, *** p < 0.05, 0.01 or 0.001 relative to Wt/Wt. One-way ANOVA followed by Student-Newman-Keuls test and Student's t-test were used for statistical analysis. Average ± SEM

5.9 Dopamine release, uptake and amphetamine responses in GDNFh hypermorphic mice (Study III)

In dSTR both dopamine release and uptake were increased in GDNFh mice, when measured by cyclic voltammetry and *in vivo* chronoamperometry respectively (Figure 5.7a, b). Remarkably, while in chronoamperometry wild-type dopamine uptake rate was quickly saturated, when higher amounts of dopamine were ejected to striatum this did not happen in GDNFh mice (Figure 5.7b). Enhanced dopamine uptake was accompanied by increased locomotor activity and striatal dopamine release in response to amphetamine in GDNFh mice (Figure 5.7c, d). Amphetamine also appeared to deplete synaptic dopamine vesicles faster in striatal slices of GDNFh mice (Figure 5.7e). Together this indicates that dopamine terminal function in the striatum is greatly altered due to higher levels of endogenous GDNF.

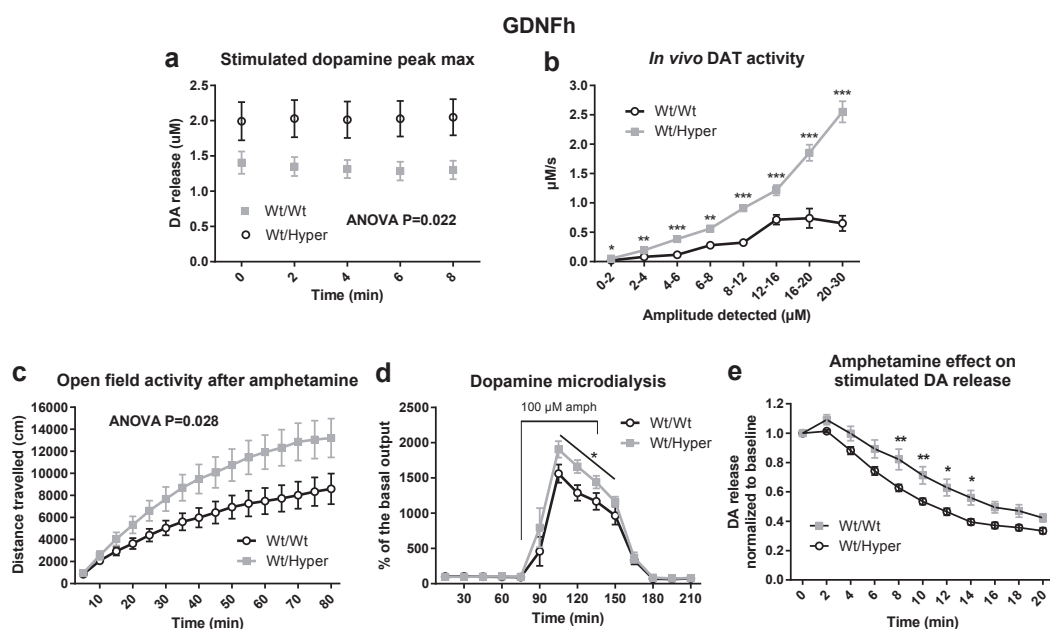


Figure 5.7. Dopamine release, uptake and amphetamine responses in GDNFh mice. (a) Striatal dopamine terminal stimulation consistently produced higher dopamine peaks in striatal slices of GDNFh mice, when measured by cyclic voltammetry. (b) Dopamine uptake was found to be elevated in chronoamperometric study, where escalating doses of dopamine were injected to the striatum of anaesthetized animals. (c) Amphetamine injection (1 mg/kg; i.p.) caused enhanced locomotor response in GDNFh mice compared to their Wild-type littermates. (d) Local amphetamine stimulation caused higher elevation in extracellular dopamine levels in GDNFh mice, when measured by *in vivo* microdialysis in freely moving animals. (e) Amphetamine depleted synaptic dopamine vesicles faster in GDNFh mice according to cyclic voltammetry measurements on striatal slices. DAT-dopamine transporter. *, **, *** $p < 0.05$, 0.01 or 0.001 relative to Wt/Wt. One-way ANOVA for repeated measures (followed by Bonferroni post hoc test for chronoamperometry) and two-way repeated measures ANOVA (followed by Sidak's multiple comparisons in the voltammetric amphetamine analysis). Error bars are mean \pm s.e.m.

5.10 Elevated GDNF protects dopamine system from lactacystin-induced neurotoxicity (Study III)

As dopamine uptake is 5-fold stronger in GDNFh mice, we found the GDNFh mice to be 5-fold more sensitive to DAT-dependent neurotoxin 6-hydroxydopamine (6-OHDA) compared to their wild-type littermates (Study III, Figure 4P, Q). So in order to study neuroprotection we needed to utilize a model that is independent of DAT function. We therefore utilized proteasome inhibitor lactacystin, which causes accumulation of unfolded proteins and dopaminergic degeneration, when injected above SNpc (Bentea et al., 2015). In our experiment unilateral lactacystin delivery (4 µg) just above SNpc caused clear side bias in the corridor test (Grealish et al., 2010) 4 weeks after the injection in wild-type mice but not in their GDNFh littermates (Figure 5.8a). Consistent with our experience that a functional lesion requires at least ~50 % loss in striatal dopamine, the dSTR dopamine levels were reduced by ~60 % in wild-type mice and by only ~25 % in GDNFh at 5 weeks post lesion (Figure 5.8b). In addition, dopamine metabolites DOPAC and HVA were better preserved in GDNFh mice (Study III, Figure 4S). However, the reduction in SNpc TH+ cell numbers was approximately the same in both genotypes (30-40 %) (Figure 5.8c). This indicates a protective or restorative function for endogenous GDNF that still did not save the dopaminergic cell bodies that reside in the SNpc (Figure 5.8c).

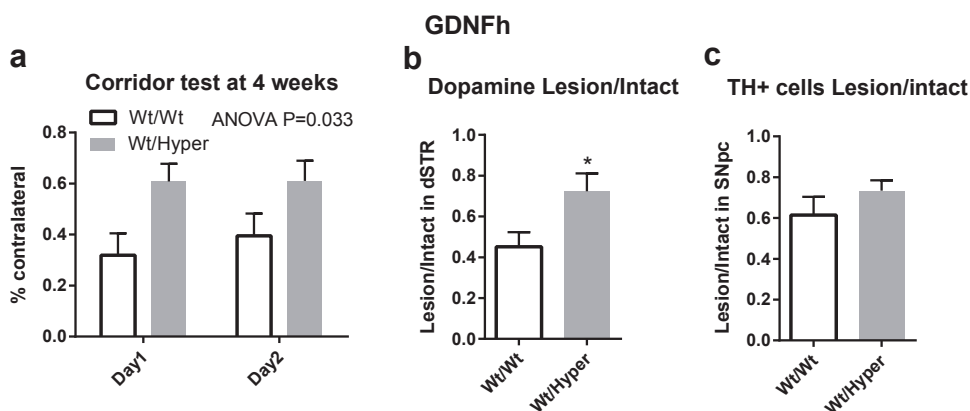


Figure 5.8. GDNFh mice are protected from lactacystin-induced neurotoxicity. (a) 4 weeks after supranigral lactacystin injection Wild-type mice demonstrated a clear ipsilateral side bias in corridor test, while GDNFh mice did not display any bias. (b) 5 weeks after lactacystin Wt dopamine levels in the dSTR were reduced almost 60 % in the injected side, while in GDNFh mice this reduction was a bit over 20 %. (c) The number of SNpc TH+ cells was reduced to a similar extent in both genotypes. TH-tyrosine hydroxylase; SNpc-substantia nigra pars compacta; dSTR-dorsal striatum. *, $p < 0.05$ relative to Wt/Wt. Student's t-test and repeated measures ANOVA were used for statistical analysis. Error bars are mean ± s.e.m.

Table 5.3. Summary of findings in GDNF cKO and GDNFh adult mice

Measure	GDNF cKO	GDNFh
Brain GDNF expression	↓↓	↑
SNpc dopamine cells	↔	↑
Striatal dopamine	↔	↑
Response to amphetamine	↓	↑
Dopamine release	↔	↑
Dopamine uptake	↑	↑↑
Striatal DAT levels	↑	↔
Sensitivity to neurotoxins	n.d.	↓

↔ indicates no change, ↑ indicates an increase, ↑↑ indicates a strong increase, ↓ indicates a decrease and ↓↓ indicates a strong decrease in comparison to Wild-type littermates. n.d. - not determined.

6 DISCUSSION

6.1 Role of GDNF in dopamine neuron maintenance during development

We found elevated SNpc TH⁺ cell numbers in P7.5 and adult GDNFh mice, while in Nestin-Cre GDNF cKO mice these numbers were unaltered at 3 months of age. Unaltered TH expression levels and similarly increased numbers of VMAT2 stained SNpc neurons in GDNFh mice suggest that the detected increase is not due to upregulation in TH, but by actual increase in dopamine neuron numbers. The natural cell death of dopaminergic neurons takes place in two apoptotic waves that peak at P2 and P14 in mice (Jackson-Lewis et al., 2000). The unaltered SNpc dopamine neuron numbers at 3 months old Nestin-Cre GDNF cKO mice suggest that GDNF alone is not necessary for the maintenance of these neurons during their development and postnatal maturation. However, the increased cell numbers in GDNFh mice indicate that endogenous GDNF can affect the number of neurons surviving this cell death period. An alternative explanation is that GDNF stimulates neurogenesis of dopamine neurons. Hence, it appears that other growth factors or some other mechanisms can compensate for the absence of GDNF during dopamine neuron development and maturation.

As TH⁺ cell numbers were already elevated at P7.5 in GDNFh mice and remained similarly increased at 3 months of age, it appears that endogenous GDNF plays a role during the first but not the second phase of natural cell death. If endogenous GDNF also played a role in the second phase, there would have been an even larger difference in cell number at 3 months of age between GDNFh mice and their wild-type littermates. A specific role for GDNF in the first phase of natural cell death has been previously suggested based on studies with striatally delivered exogenous GDNF (Oo et al., 2003; Kholodilov et al., 2004). In contrast to our study, when GDNF was transgenically expressed in the striatum, SNpc TH⁺ cell numbers were only transiently elevated at P7-9 and this increase did not persist to adulthood (Kholodilov et al., 2004). This difference highlights the importance of correct spatial and temporal pattern of GDNF expression for its full effects.

Our results with the Nestin-Cre GDNF cKO mice are in perfect alignment with the results from Nestin-Cre BDNF cKO mice; injection of BDNF neutralizing antibodies to SNpc (Oo et al., 2009) or GDNF neutralizing antibodies to dSTR (Oo et al., 2003) at P5-6 both amplify the apoptotic cell death of SNpc dopamine neurons. However, the conditional deletion of brain BDNF with Nestin-Cre did not have this effect (although SNpc organization was disrupted) (Oo et al., 2009), similarly to what we report using Nestin-Cre GDNF cKO mice. Furthermore, the number of TH⁺ cells was unchanged in mice with Ret-deficient dopamine neurons at 3 months of age (Kramer et al., 2007), but increased in MEN2B mice with constitutively active Ret (Mijatovic et al., 2007). Taken together it appears that the absence of endogenous GDNF, BDNF or Ret can be compensated during dopamine neuron development and maturation. However, increasing the endogenous GDNF expression or significantly enhancing Ret signaling leads to increased numbers of SNpc dopamine neurons surviving the natural cell death period. This supports the hypothesis that endogenous GDNF functions as a target-derived neurotrophic factor for SNpc dopamine neurons during their development, but also indicates that GDNF is not indispensable in this role. This further suggests that many neurons are responsive for increases in neurotrophic factor levels, but not dependent on them, at least of any single one. The fact that most intracellular pathways are shared between different growth factors is in line with this idea.

6.2 Impact of endogenous GDNF on striatal dopamine levels and release

Similarly to the SNpc dopaminergic cell numbers, the striatal levels of dopamine were elevated in GDNFh mice at P7.5 and at 3 months of age, but unchanged in 3 months old Nestin-Cre GDNF cKO mice. In 3 months old GDNFh mice dopamine and DOPAC levels were elevated specifically in dSTR, but not in vSTR. Furthermore, the number of DAT+ terminals was elevated in dSTR of GDNFh mice. Together, increased dopamine cell and terminal numbers as well as tissue levels of dopamine suggest that stronger GDNF signal leads to an enlarged nigrostriatal dopaminergic system. However, an enhanced GDNF signal might additionally stimulate dopamine synthesis and storage as happens in MEN2B mice, where the elevation in dSTR dopamine levels is much higher than the increase in SNpc TH+ cells or dSTR DAT+ terminal numbers (Mijatovic et al., 2007). This hypothesis is supported by the fact that the dSTR dopamine levels are elevated a bit more than the SNpc cell numbers in GDNFh mice (~25 % vs. ~15 %).

Exogenous GDNF reduces TH levels but increases TH phosphorylation leading to enhanced dopamine synthesis (Salvatore et al., 2004, 2009). Therefore, dopamine synthesis in GDNFh mice can be increased even though total TH levels were unchanged. VMAT2 packs dopamine to synaptic vesicles, so its activity directly affects vesicular dopamine content (Eiden and Weihe, 2011; German et al., 2015), and vesicular packing also relieves TH from the feedback inhibition by dopamine (Tekin et al., 2014). Hence, investigating VMAT2 activity and TH phosphorylation in GDNFh mice might further resolve the mechanisms of how endogenous GDNF increases striatal dopamine levels. The results from Nestin-Cre GDNF cKO mice are in line with Ret cKO crossed DAT-Cre mice, where the striatal levels of dopamine and its metabolites were similarly unaltered (Kramer et al., 2007). In conclusion, higher levels of endogenous GDNF lead to an enlarged nigrostriatal dopaminergic system during development and maturation and possibly to higher dopamine storage levels in the dorsal striatum. To the contrary, brain GDNF absence does not alter the nigrostriatal system size or striatal dopamine levels.

In parallel to dSTR dopamine levels, we found striatal dopamine release unchanged in Nestin-Cre GDNF cKO mice, but enhanced in GDNFh mice. The tissue dopamine levels most likely explain why dopamine release is enhanced in GDNFh mice, but not in Nestin-Cre GDNF cKO; as GDNFh mice appear to have more dopamine in the striatal synapses, an electrical stimulus would cause more dopamine to be released. The increased dSTR tissue levels of the dopamine primary metabolite DOPAC suggest that dopamine release is naturally stronger in GDNFh mice. Alternatively, increased DOPAC levels may simply reflect the higher dopamine storage levels, which augment vesicular leakage and consequent cytoplasmic metabolism of dopamine (Eisenhofer et al., 2004). In support of our data, exogenous GDNF has been shown to increase vesicular dopamine content and its subsequent release in vitro (Pothos et al., 1998) as well as potassium evoked dopamine release in vivo (Hebert et al., 1996). To test whether increased tissue dopamine levels indeed enhanced dopamine release, dopamine release properties could be measured also in GDNFh vSTR, where the tissue dopamine levels remained unaltered.

Neurotransmitter-containing vesicles are divided into three major vesicle pools: A readily releasable pool, a recycling pool and a reserve pool (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010). The different synaptic pools are in a dynamic balance between each other, but the speed of mixing is variable. Studying the absolute and relative changes in the different dopamine

vesicle pools in GDNFh mice would uncover how endogenous GDNF affects dopamine vesicle trafficking and recycling. This could further explain the mechanisms of increased dopamine release. Indeed, results with MEN2B mice suggest them having larger releasable and storage pools (representing combined recyclable and release pools) for dopamine (Mijatovic et al., 2008). However, in MEN2B mice the levels of striatal TH and DAT were elevated and dopamine levels increased also in the vSTR (Mijatovic et al., 2007), while in GDNFh mice TH and DAT levels were unchanged and vSTR dopamine levels were normal. The dopaminergic phenotype obviously qualitatively differs between GDNFh and MEN2B mice, so direct comparisons should be made with caution.

In conclusion, enhanced endogenous GDNF expression increases striatal dopamine release most likely reflecting increased tissue dopamine levels. Absence of brain GDNF has no impact on these parameters.

6.3 Role of GDNF in dopamine neuron maintenance during aging

In order to resolve the impact of complete GDNF-deficiency on midbrain dopamine neuron maintenance during normal aging we stereologically estimated SNpc TH+ cell numbers at 14 and 19 months of age in the GDNF cKO Nestin-Cre line. To our surprise, there was no reduction in SNpc TH+ cell numbers in mice lacking one (C/KO) or both (F/KO + Nestin-Cre) *Gdnf* alleles. The TH+ cell numbers in VTA were unaltered and the TH-stained midbrain area seemed morphologically normal. Moreover, striatal TH+ innervation (optical density) was unchanged in the target areas of these neurons, dSTR and vSTR (Study I, Figure 1g). In 17 months old GDNFh mice SNpc TH+ cell numbers ($P = 0.034$) and dSTR dopamine levels ($P = 0.0054$) were still significantly elevated (~10-15 %) compared to wild-type littermates (unpublished results). This increase is slightly lower in magnitude, compared to the young animals where it was ~15-25 %. It suggests that some of the extra neurons these mice had were lost during aging and possibly that the effects of GDNF on dopamine storage levels were also weakened in old mice. Further studies will be needed to resolve this issue. In any case, the enhanced GDNF expression in GDNFh mice did not protect additional dopamine neurons from the potential age-related loss compared to their wild-type littermates. Together, our data strongly indicate that endogenous GDNF is not necessary for the maintenance of midbrain dopamine neurons during aging. This is in clear contrast with results in (Boger et al., 2006) that reported age-related reduction in TH+ cell numbers and TH+ striatal innervation in mice lacking just one functional GDNF allele. However, another group did not see these degenerative changes in aged GDNF heterozygous mice or even in double heterozygous mice that lacked one allele for both *Gdnf* and *Tgfb2* (Heermann et al., 2010). Our results resolve this existing controversy by demonstrating that lifelong lack of one or two functional GDNF alleles in the brain does not compromise the integrity of nigrostriatal dopamine system during aging. Our findings are in line with the aged *Ret* cKO mice, where degeneration of the nigrostriatal system was moderate at 24 months (38 % TH+ cell loss) (Kramer et al., 2007). As *Ret* serves as a common signaling receptor for all the GDNF family ligands (Airaksinen and Saarma, 2002), it is very likely that NRTN for example provided enough *Ret*-mediated trophic support in our GDNF-deficient mice that the nigrostriatal dopamine system maintenance was not compromised.

All the mouse lines mentioned above carry their GDNF or Ret mutation since early embryonic development. Thus, it is possible that developmental compensatory mechanisms in the dopamine systems have permanently reduced their inherent dependency on GDNF/Ret signaling (Ibáñez, 2008). In order to test this hypothesis we utilized two additional cKO approaches, where GDNF deletion was induced in young adults (2 months of age) to avoid any possible developmental compensation. Unilateral AAV5-Cre injection to the striatum removed GDNF specifically from the dSTR, which is the innervation target for the SNpc dopamine neurons. *Gdnf* mRNA expression was brought down to almost undetectable levels by 7 months post injection, but this did not cause any changes in SNpc TH+ cell numbers or dSTR dopamine levels compared to the non-injected side or to the control mice. Similarly, adult *Gdnf* reduction in the *Esr1*-Cre GDNF cKO line did not affect the SNpc TH+ cell numbers.

Our results are in striking contrast with the paper that reported dramatic degeneration in both SNpc and VTA dopaminergic cells after reducing GDNF just to 40 % of wild-type levels. It is important to note that GDNF heterozygous mice, which they and we used as controls, are reported to have about 50-60 % of wild-type GDNF levels (Boger et al., 2006; and our published and unpublished results). In correspondence Pascual and colleagues recently suggested that it is this small drop from 50-60 % to 40 % in GDNF levels that makes the difference of life and death to brain catecholaminergic neurons. In our *Esr1*-Cre line GDNF mRNA or protein levels dropped down to ~10-15 % of the wild-type levels and even more in 9 months old AAV5-Cre injected mice, but there was still no reduction in SNpc dopaminergic cells. To our surprise, the reduction in *Esr1*-Cre GDNF cKO *Gdnf* mRNA levels appeared to happen independently of TMX injections, but still after 2 months of age, as at that point there was no reduction yet (Study I, Figure 3b). Indeed, the ‘leakiness’ of Cre in *Esr1*-Cre line has been reported before (Liu et al., 2010) and in our case this should not make a significant difference as the model achieved its purpose (adult reduction in *Gdnf* expression) and the results were in line with the complementary approach with AAV5-Cre. One suggested explanation to the large discrepancy between the two studies is the different genetic background of the mice used: C57bl6/129Sv in (Pascual et al., 2008) and 129Ola/ICR/C57bl6 in our study. Hence, they used a mix of two strains while we used a mix of three. The possibility of a dramatic background effect cannot be ruled out, but one may ask how generalizable a result is, if it does not repeat in another mixed mouse line. Another possible, but unlikely, explanation is that direct TMX toxicity contributed to the neurodegenerative phenotype reported in (Pascual et al., 2008), as the doses administered were high. Further studies will be needed to fully resolve this discrepancy. However, together our results indicate that reductions in GDNF levels are not associated with dopaminergic degeneration. This is supported by the fact that mutations in the *Gdnf* coding region are not commonly contributing to human PD pathogenesis, although this might simply be due to lethality of such mutations.

6.4 Effects of endogenous GDNF on dopamine uptake

To examine the effects of endogenous GDNF on striatal DAT function we studied dopamine reuptake and responses to amphetamine stimulation in GDNF cKO Nestin-Cre and GDNFh mouse strains. Both strains had enhanced dopamine reuptake, while amphetamine-stimulated hyperlocomotion was reduced in Nestin-Cre GDNF cKO mice and augmented in GDNFh mice. Interestingly, while GDNFh mice had no changes in striatal tissue levels of DAT, dSTR DAT levels were increased in GDNF cKO Nestin-Cre mice. Moreover, in GDNF cKO Nestin-

Cre mice a larger fraction of DAT appears to be located in the plasma membrane, where it can contribute to the reuptake of extracellular dopamine. Together, increased DAT expression and its augmented localization to the plasma membrane likely explain enhanced dopamine uptake in the absence of brain GDNF, although other, yet unknown, mechanism(s) may also contribute. Even though the cellular localization of DAT has not been determined yet in GDNFh mice, it appears that their enhanced DAT function is at least partially due to different mechanism than in GDNF cKO Nestin-Cre mice, as discussed below.

Amphetamine increases cytosolic dopamine levels by releasing dopamine from the storage vesicles, which eventually induces DAT-mediated dopamine efflux to the extracellular space (German et al., 2015). Amphetamine also directly inhibits DAT function (German et al., 2015), which contributes to the increased extracellular dopamine levels and resulting hyperlocomotion. It appears that amphetamine had fundamentally different effects in the two GDNF mouse strains. In GDNFh mice amphetamine depleted dopamine from the storage vesicles faster, while in Nestin-Cre GDNF cKO mice this aspect was unchanged (Study II, Figure 1E). This suggests that GDNFh vesicles contain more dopamine and their VMAT2 function is enhanced. In Nestin-Cre GDNF cKO line amphetamine-induced dopamine efflux was clearly reduced, mostly because their DAT was much slower to reverse its transport direction (reduced DAT reversal time). This could be because the larger surface DAT population would respond slower to amphetamine, if a significant fraction of DAT molecules remained in the uptake transport mode. In GDNFh mice, DAT reversal time appeared to be unchanged (unpublished data). Finally, *in vivo* microdialysis showed enhanced extracellular dopamine levels after amphetamine stimulation in GDNFh mice (published), but no differences in GDNF cKO Nestin-Cre (unpublished data).

Together these results suggest that altered locomotor response to amphetamine in the two mouse strains (increased in GDNFh and decreased in GDNF cKO Nestin-Cre) is due to different mechanisms. In GDNFh mice it may have been due to faster dopamine depletion from the storage vesicles and in Nestin-Cre GDNF cKO because of slower DAT reversal or otherwise altered DAT function. The reduced DAT reversal time in GDNF cKO Nestin-Cre only, further suggests that even though dopamine reuptake was robustly increased in both mouse models, the underlying mechanism was different. For example, in GDNFh mice increased DAT activity may simply be a secondary compensatory mechanism to increased dopamine release. On the other hand, both PI3K/Akt and MAPK pathways that are downstream from Ret, are known to positively regulate DAT function (Eriksen et al., 2010).

Literature suggests several more direct interactions and relationships between GDNF and DAT, probably reflecting the complexity of their interaction. Already the first GDNF article reported that exogenous GDNF stimulates dopamine uptake in cultured embryonic dopamine neurons (Lin et al., 1993). In contrast, a recent study reported that GDNF/Ret signaling reduces DAT *in vitro* and *in vivo* activity by promoting its removal from the plasma membrane (Zhu et al., 2015). This interaction was reported to depend on endocytosis mediator Vav2, as Ret activation enhanced binding between Vav2 and DAT, and to be specific for vSTR (Zhu et al., 2015). In another recent study viral over-expression of GDNF in dSTR reduced dopamine uptake (Barroso-Chinea et al., 2016). Decrease in DAT activity was reported to be associated with DAT-DAT interactions and DAT interactions with α -synuclein (Barroso-Chinea et al., 2016). Interestingly, increased DAT activity has been reported in MEN2B mice with greatly enhanced Ret signaling (Mijatovic et al., 2008) as well as in mice heterozygous for GDNF (Littrell et al., 2012) and Ret (Zhu et al., 2015). Finally, it is important to note that we did not observe significantly increased dopamine uptake in heterozygous C/KO mice that served as controls in

our study. Our data suggests that absence of one *Gdnf* allele is not sufficient to alter DAT activity, although DAT expression was slightly increased.

It is possible that several of the previously identified mechanisms are in a complex interplay in our mouse lines resulting in enhanced dopamine uptake after both abolished and enhanced GDNF expression. In contrast to Zhu et al. we found DAT cell surface localization increased in dSTR, but unchanged in vSTR (unpublished data), while they reported completely opposite effect in Ret heterozygous mice (Zhu et al., 2015). The apparent complexity and importance of the GDNF/DAT relationship calls for further research. Another factor that potentially confounds the resolution of GDNF's role in DAT regulation is that in GDNFh and Nestin-Cre GDNF cKO mice GDNF expression is changed since the early embryonic stage. This can cause fundamental alterations in midbrain dopamine systems during their development and maturation, which may influence DAT function in the adult animals. In order to differentiate between developmental and direct GDNF effects, the reduction or increase in endogenous GDNF levels should be induced in mature adult animals, for example by AAV5-Cre injection.

In conclusion, both increase and decrease in endogenous GDNF expression lead to enhanced dopamine uptake by apparently different mechanisms. However, locomotor response to amphetamine appears to parallel changes in endogenous GDNF expression, but also here the mechanism is likely different in the two mouse models. The complex interaction between GDNF and DAT most likely contributes to these phenotypes. Further research is required to resolve the effects of endogenous GDNF on dopamine uptake and on dopamine terminal function in general.

6.5 Role of endogenous GDNF in neuroprotection

As dopamine uptake was greatly enhanced in GDNFh mice we could not use the traditional dopaminergic neurotoxins like 6-OHDA and MPTP to study neuroprotection, as their toxicity to dopamine neurons is dependent on DAT activity (Duty and Jenner, 2011). In line with that expectation, GDNFh mice had enhanced sensitivity to striatal 6-OHDA injection (Study III, Figure 4P-Q) and therefore we needed to utilize a neurotoxin whose effects are DAT-independent. We chose proteasome inhibitor lactacystin, whose toxicity is based on inhibition of proteasomal protein metabolism and subsequent cellular accumulation dysfunctional proteins (Duty and Jenner, 2011). This mechanism appears to bear direct relevance to human PD pathology, where accumulation and spread of proteinous Lewy bodies seems to play a key role (Meissner et al., 2011; Goedert et al., 2013).

Four to five weeks after supranigral lactacystin delivery, GDNFh mice exhibited no motor function impairment in corridor test and better-preserved dopamine levels in dSTR compared to their wild-type littermates. However, SNpc TH+ cells were similarly reduced in both genotypes. This suggests that endogenous GDNF is able to protect or regenerate striatal dopaminergic fibers, but does not protect the nigral cell bodies. There are several mutually non-exclusive explanations for the higher dSTR dopamine levels we observed 5 weeks post lesion: Increased endogenous GDNF expression either helped more dopaminergic fibers to survive the toxic insult, promoted their sprouting and recovery after the acute lesion or stimulated dopamine production in the remaining terminals. Results from MPTP lesioned Ret cKO mice support the second hypothesis, as there was no difference in the initial lesion severity, but the Ret-deficient dopamine neurons were incapable for axonal resprouting (Kowsky et al., 2007). On the other hand, constitutively

active Ret was reported to be protective for SNpc cell bodies but not for axonal terminal in MEN2B mice (Mijatovic et al., 2011). However, those studies were conducted using 6-OHDA and MPTP in MEN2B mice that have greatly enhanced DAT activity (Mijatovic et al., 2008). In the light of our data with 6-OHDA in GDNFh mice, it is very likely that the MEN2B response to those toxins was a combination of increased sensitivity and a massive trophic signal through constitutively active Ret. To test this hypothesis, MEN2B mice should be studied in the same lactacystin model we used with GDNFh mice.

To further resolve the nature of the protective/restorative effect in GDNFh mice, the animals should be analyzed at one week after lactacystin injection, as at that point lactacystin lesion appears to be fully developed (Bentea et al., 2015), but possible axonal resprouting has not happened yet (Kowsky et al., 2007). In addition, Nestin-Cre GDNF cKO mice should be studied in the lactacystin model to find out whether brain GDNF absence predisposes the nigrostriatal dopamine system to its toxic effects.

In summary, endogenous GDNF displays clear functional and neurochemical benefits in the lactacystin-based Parkinson's disease model, but the exact mechanisms remain to be resolved.

6.6 General discussion

We found brain *Gdnf* mRNA expression to be either undetectable or very low in the GDNF cKO strains (Nestin-Cre, AAV5-Cre and *Esr1*-Cre) and moderately increased in the GDNFh mice. From that perspective, the models are opposite to each other and we naturally expected them to have opposite phenotypes. However, the only opposite phenotype we have found so far is the locomotor response to amphetamine and also in this case the mechanism appears to differ between these models. In most of the core dopamine system measures GDNFh mice had a phenotype, but the GDNF cKO mice did not. An important exception were amphetamine responses and striatal dopamine uptake, which are at least partially related.

Similarly to our findings with GDNF cKO Nestin-Cre mice, mutant mice for PD-related genes α -synuclein, leucine-rich repeat kinase 2 (LRRK2), Parkin, DJ-1 and PTEN-induced kinase 1 (PINK1) exhibit changes in dopamine release or uptake without accompanying loss of dopaminergic neurons or striatal dopamine content (Reviewed in Rice et al., 2011). It seems that a single mutation in one of these factors is not sufficient to cause a full parkinsonian phenotype, but maybe requires a "second hit". It is also possible that brain GDNF absence predisposes to neurodegeneration if combined with an adequate additional genetic or environmental stressor, as has been shown for Ret-deficient mice (Aron et al., 2010; Meka et al., 2015). Also in α -synuclein overexpressing mice dopamine neurotransmission is altered prior to dopamine neuron loss (Janezic et al., 2013).

However, in the big picture our data strongly suggests that at least some of the potential consequences of brain GDNF absence are compensated for, thus leading to a dampened phenotype. These sorts of limitations of the knock-out approach have been acknowledged for 20 years (Routtenberg and Gerlai, 1996). The fact that signaling receptor Ret is shared with all the GDNF family members is likely to explain why Ret-deficient mice have a stronger phenotype than GDNF cKO mice. However, trophic intracellular signaling pathways, like PI3K/Akt and ERK/MAPK, are not specific to Ret, and hence it is very likely that neurons can partially compensate for the reduced Ret signal from other sources. Recent evidence suggests that dopamine neurons can dynamically influence the amount of striatal GDNF available for them (Gonzalez-Reyes et

al., 2012). In the absence of GDNF/Ret, they might be able to stimulate secretion of some other trophic factor to maintain their survival and function. Moreover, it is safer for the neurons not to rely on a single source of survival signals during their lifecycle. The apparent limitations of gene ablation strategy call for novel animal models to study gene function.

The GDNFh strain indeed appears to be more informative regarding the role and functions of endogenous GDNF. Contrarily to reductions in GDNF expression, nigrostriatal dopaminergic neurons appear to be sensitive to a moderate increase in endogenous GDNF levels. This is also in contrast with various studies using GDNF delivery or viral overexpression of up to more than 100 fold over the natural expression levels. Our results suggest that the correct spatiotemporal pattern is more important for GDNF effects than the total amount of GDNF protein delivered to the tissue. Very strong overexpression can be expected to produce various adverse-effects that are not necessarily related to the natural GDNF signal. In line with this, increased levels of endogenous GDNF indeed did not elicit side-effects reported with ectopic GDNF applications, such as hyperactivity (Hudson et al., 1995; Hebert et al., 1996; Hebert and Gerhardt, 1997) or reduced food intake and weight loss (Hudson et al., 1995; Manfredsson et al., 2009) (Study II, Figure 4U-W). This suggests that elevation of endogenous GDNF might be a viable future strategy for treating PD. However, more basic research is needed first to understand its full potential and limitations. The ongoing studies with conditional GDNF hypermorphs (or GDNF “knock-up” mice), where GDNF 3’UTR replacement can be induced in adult animals, will be an important milestone in this path.

Results with GDNFh mice suggest that endogenous GDNF plays an important role in the development, function and protection of these neurons. Our data also indicates that *in vivo* studies using ectopic GDNF do not reliably or comprehensively predict the functions of endogenous GDNF (Study III, Table 1). However, even though we have clearly demonstrated the potential value of 3’UTR replacement method, it has its own limitations as well. The native 3’UTR might contain important signals for processing and cellular localization that will be lost upon replacement. In any case, the potential extent of misexpression would be minor compared to viral vector delivery or conventional overexpression models. The extent of the target gene derepression also depends on the amount of destabilizing miRNAs and RNA binding proteins present in each tissue. Finally, 3’UTR replacement is not feasible for many genes as already *Gfra1*, 3 and 4 do not appear to contain miRNA binding sites (unpublished data). Regardless of its limitations, 3’UTR replacement method is likely to greatly advance the understanding of *in vivo* functions of many important genes.

7 CONCLUSIONS

The aim of these studies was to gain insight into the role of endogenous GDNF in the development, function and maintenance of midbrain dopamine neurons. The principal findings of the study are:

1. Endogenous GDNF functions as a target-derived neurotrophic factor for SNpc dopamine neurons during their development and maturation, but it is not indispensable in this role. Augmented endogenous GDNF expression leads to larger nigrostriatal dopamine system during development.
2. Endogenous GDNF alone is not required for the maintenance of midbrain dopamine neurons during aging.
3. Elevated levels of endogenous GDNF lead to increased dopamine content and dopamine release in the dSTR, whereas brain GDNF absence had no effect on them. Both an increase and a decrease in GDNF expression lead to enhanced dopamine uptake.
4. Augmented endogenous GDNF expression provides clear functional and neurochemical protection in a neurotoxin-based Parkinson's disease model.

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A handwritten signature in black ink, appearing to read 'Jukka', with a stylized, cursive script.

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